Table of Contents

LARGE-SCALE CsCl preparation of Bacterial genomic DNA

1. Grow 100 ml culture of bacterial strain to saturation.
2. Pellet cells for 10 min at 6000 rpm (use GSA rotor)
3. Resuspend cells gently in 9.5 ml TE buffer. Add 0.5 ml of 10% SDS a
   50 ul of 20 mg/ml proteinase K. Mix thoroughly and incub
   1 hr at 37°C.
4. Add 1.8 ml of 5M NaCl and mix thoroughly.
5. Add 1.5 ml CTAB/NaCl solution. Mix thoroughly and incu
   20 min at 65°C. (can stay for a moment)
6. Add an equal volume (13 ml) of chloroform/isoamyl alc
   Extract thoroughly. Spin 10 min at 7000 rpm (use SS34
   , room temperature. to separate phases.
7. Transfer aqueous supernatant to a fresh tube using a
   wide-boreel pipet.
8. Add an phenol/chloroform/isoamyl alcohol. Extration the
   equal volume
   Repeat three times.
9. Add an equal volume of chlorform/isoamyl alcohol.
10. Add 0.6 volume isopropanol and mix. (-20°C overnight)
    (-20°C can help to precipitate)

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Notebook Number: _____

Date: 07/28/99

Continue:

11. Transfer the precipitate to 1 ml of 70% ethanol in a fresh tube (just brown-red one), by hooking it on the end of a Pasteur pipet that has been bent (by heating it) and sealed.

12. Drying the pellet (just in the air). Remove ~~white~~ white and stringy DNA to the 2nd TE buffer.

13. Do the electrophoresis of nucleic acid (there are a lot of DNA and RNA)

14. Use Rnase to digest the genomic DNA overnight.

Genomic DNA. (continue)

1. Use EcoR I to do restriction. put { genomic DNA 77ul; buffer (H) 9ul; EcoR I 4ul. } Digest genomic DNA. overnight.

2. do the electrophoresis at 60 V.
put 109 ul. digested DNA (90 ul. digested DNA 19ul dye)
the result is not good.

Extracting Plasmid DNA of E.coli. (Qiagen Midi and Maxi Prot.)

1. Inoculate a 5-ml LB medium with E. Coli. 37°c. shake. to afternoon.

2. Inoculate 200ml medium. Grow at 37°c ~~for~~ overnight.

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Extracting plasmid DNA. (continue)

3. Harvest the bacterial cells by centrifugation at 6000g for 15min at 4°C.
4. Resuspend the bacterial pellet in 4 ml of buffer P1.
5. Add 4ml of buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate at room temperature for 5 min.
6. Add 4 ml of chilled buffer P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 15 min.
7. Centrifuge at 20000 g for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.
8. Re-centrifuge the supernatant at ~~x~~20,000 xg for 15 min at 4°C Remove supernatant containing plasmid DNA promptly. ~~Alternatively, the sample can be fill~~
9. Equilibrate a QIAGEN-tip 100 by applying 4ml Buffer QBT. and allow the column to empty by gravity flow.
10. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
11. Wash the QIAGEN-tip with 10ml (two times) buffer QC.
12. Elute DNA with 5 ml buffer QF.
13. precipitate DNA by adding 3.5 ml room-temperature isopropanol to the eluted DNA. Mix and Centrifuge immediately at ~~15~~ 13000 rpm & for 30min at 4°C. Carefully decant ~~the~~ the supernatant.
14. Wash DNA pellet with 2ml of room-temperature 70% ethanol, and centrifuge at ~~15000~~ 13000 rpm & for 10min. Carefully decant the supernatant without

disturbing the ~~pelle~~ pellet.

15. Air-dry the pellet for 5-10 min. and redissolve the DNA in a suitable volume of buffer. → (TE buffer → 1/2 ml)

16. Do electrophoresis to check it. → to digest? → next page

8/3/99

High-efficiency transformation by electroporation

1. Inoculate a single colony of E Coli cells into 5 ml LB medium. Grow 5 hr to overnight at 37°c with moderate shaking

2. Inoculate 2 ml of the culture into 500 ml LB medium in a ster. 1000 flask. Grow at 37°c. ~~shaking at~~ in ~~incubator~~ shaker to an OD_{600} of ~~0.5 and 0.7~~ 0.25. (if > 0.7 need more time)

3. Chill cells in an ice-water bath 10 to 15 min and transfer to a prechilled 50ml centrifuge bottle.

4. Centrifuge cells ~~20 min~~ 10 min at ~~6000~~ 8000 rpm. 2°c

5. Pour off supernatant and ~~respend~~ resuspend the pellet in ice-cold water. Add 25ml ice-cold water and mix well. Centrifuge cells as in step 4 at 10 min 8000 rpm. repeat 2 times. (throw away the supernatant to the bleach bottle including)

6. Pour off supernatant immediately and resuspend the pellet by swirling in remaining liquid.

7. Add another ml ice-cold water, mix well, and centrifuge again in step 4

8. Pour off supernatant immediately and resuspend the pellet by swirling in remainning liquid.

9. Put remainning liquid to the eppendorf tube. centrifuge it at 12000 rpm 5 min. pour off by using vacuum

8. add 100 ul water to the tube. mix well. put it on ice.

(余分な水分)

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4 6

Notebook Number: _____

Date: 08/03/99

×10. Set the electroporation apparatus to 2.5 kV, 25 uf. Set the pulse controller to 200 or 400 ohms? (Under the machine instraction)

11. Add 1 ul plasmid DNA (PGP 704) in and 20 ul bacterial to tubes containning fresh or thawed cells (on ice). Mix by tapping the tube or by swirling the cells with the pipettor.

12. Transfer the DNA and cells into a cuvette that has been chilled 5 min on ice. Shake slightly to settle the cells to the ~~bottom~~ between, and wipe the ice and water from the cuvette with a Kimwipe

13. Place the cuvette in to the sample chamber.

14. Apply the pulse by pushing the button of flipping the switch.

15. Remove the cuvette. ~~Immediately add~~ LB broth immediately ~~SOC medium~~ and transfer it to a ster culture tube with a Pasteur pipet. Incubate ~~37°C~~ 60 min with moderate shaking 37°C including LB broth

16. Plate aliquots of the transformation culture on LB plates containing antibiotics. 100 ul

08/03/99

plasmid digest

PGP ~~704~~	1 ul
10× buffer	1 ul
DNase free H2O	7.5 ul
ECoR I	0.5 ul.

37°C 2 hr. → 65°C. for 15 min

run 5 ul on gel

dilution 10^{-6} 100 ul to LB OAP

10^{0} 10^{-2} 100 ul to LB OAP OAP

result

10^{0} 10^{-2} zero 2-3 v

10^{-6} 100 colonies

To electrophoresis.

1. 15 ul marker (1kb) first line
2. 65 ul digested DNA + 13 ul 6x dye. Second line.
3. Cut out 7-10 kb gene.
4. elute into Bio-rad - promega.

Fragment Isolation Protocol from Nu Sieve Gel.

1. Place the electrophoresed gel on the UV transillumination and view under long wave light. Locate the fragment of 7-10 kb and cut out the band using a glass cover slip and place the gel fragment into a 1.5ml microcentrifuge tube.
2. Heat to 65°c until gel slice melts
3. Add 3 volumes (120 ul) of BioRad DNA isolation binding buffer and for every 2 ug of DNA add 5 ul (30 ul matrix) of matrix.
4. Attach a clean 3cc syringe to a promega spin column and add the solution to it.
5. Push this slowly through the column (1 drop/3 sec)
6. Detach column from syringe and remove plunger.
7. Wash with 2 mls of 95% isopropanol.
8. Spin at 12,000 rpm for 20 sec.
9. Dry Column in Hybridization oven for a few minutes (65°C in ↑) at 65°C
10. Add 30-50 ul of water or TE. wait 1 min and spin again at 12,000 rpm for 20 sec.
11. Run 10 ul of the eluant on a gel to check recovery.

SMD

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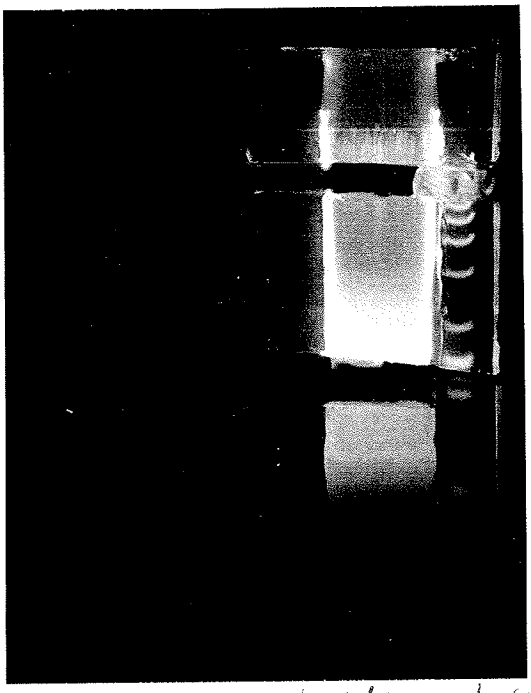
Notebook Number: _____

Date: 28/04/99

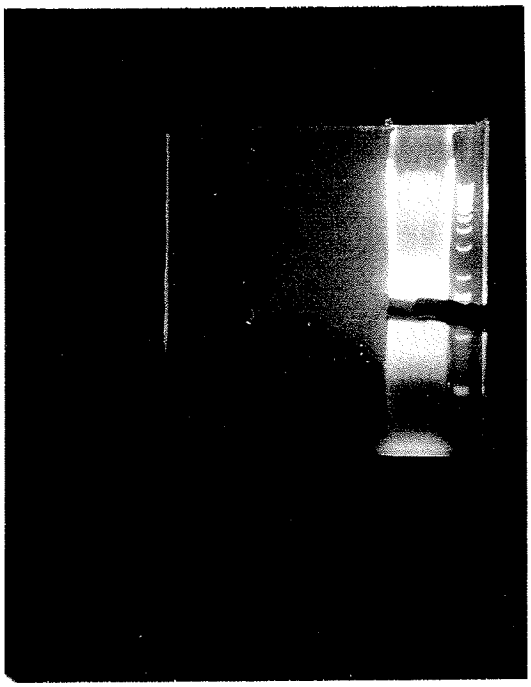


6
5
4
3
2
1 → bright

cut out 7-10 Kb

1 Kb

digested ΔSD marker (1Kb)



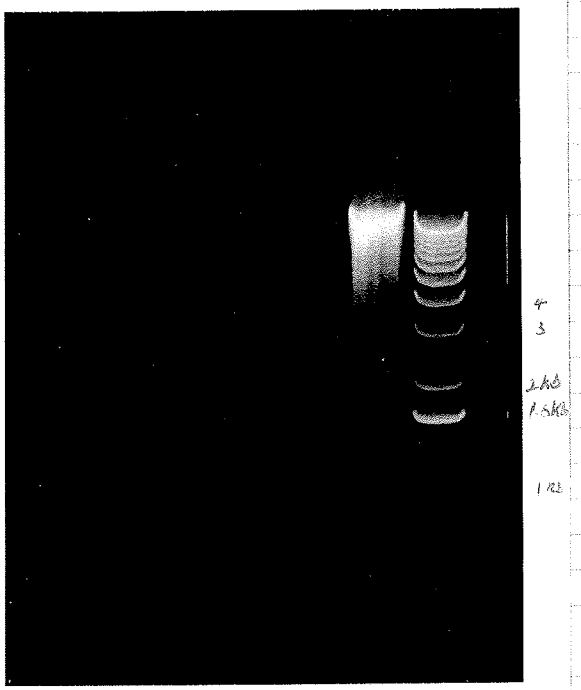
6
5
4
3
2
1

Do electrophorisis to check the result.

DNA. 5ul. + 1ul dye.

15ul Marker → 60v. run

Results:



Conjugation:

1. Donor Strain (E. Coli MGN617 PGP704 or MGN617 PLOF Km) are grown overnight with shaking at 37°C in 2ml LB broth containing 200 ug/ml ampicillin and 50 ug/ml kanamycin. (DAP)
2. Grown P. multocida 11039 overnight with shaking at 37C.
3. 50 ul MGN617 ~~or 50 ul M~~ + 100 ul 11039 to 5ml of 10mM $MgSO_4$
4. Vortex for a few seconds.
5. Transfer to a 5ml disposable syringe and filter through a 25 mm Mitricel filter.
6. Drain the filter and carefully remove it from the filter ease with sterile forceps (curved tip work best)

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10 50 ul → 5 ml broth

100 ul IPTG
200 ul
+50 ul DAP ?

Notebook Number:
Date: 08/05/99

7. Place the filter on a LB plate cell side ~~up~~ up.
(avoid bubbles between the filter and the agar)

PLOF Km
2.36V.

8. Incubate at 37 c for 8 - 18 hrs.

08/06/99

~~Results~~ 9. Resuspend the filter using 3 ml 10 mM $MgSO_4$. Vortex.

PGP 704	BHI Ap	100 ul	10^0	} negative
	BHI ApK	100 ul	10^0	
	BHI	100 ul	10^{-5}	
	LB ApDAp	100 ul	10^{-5}	
PLOF-Km	BHI ApKm	100 ul	10^0	→ negative
	BHI Km	100 ul	10^0	
	BHI	100 ul	10^{-5}	
	LBApKm DAp (200 ul)	100 ul	10^{-5}	

Transformation by electroporation

PGP 704.

PLOF Km 10^0 10^{-1} 10^{-2} delution

↓

Results

08/09/99

Digest~~ion~~:

93-146 genomic DNA (CL phenol/ch... prep)

heat incubate at 70°C 30min

Restriction Digest~~ion~~ EcoRI

DNA 51 ul
10x buffer 6
EcoRI 3

37°C 4h. → put it in 65°C water bath

08/10/99

Pb electrophoresis

903-146 genomic DNA (after digest) 60 ul

dye (6x) 10 ul

↓

run gel. (marker 12 ul)

Fragment Isolation Protocol from Gel. (See Page 7)

1. Cut out 7-10 kb and place the gel fragment into microcentrifuge tube.
2. Heat to 65°C until gel slice melts.
3. Add 3 volumes (630 ul) of BioRad DNA isolation binding buffer ~~add 630~~. (1 ug ≅ 1 ul volume) and usually add 30 ul matrix.
4. Attach a clean 3 cc syringe to a promega spin column and add the solution to it.
5. Push this slowly through the column.
6. Detach column from syringe and remove plunger.
7. Wash with 2 mls of 95% isopropanol.
8. Spin at 12,000 rpm for 20 sec.
9. Dry Column in Hybridization oven for a few minutes.
10. Add 30 ul of TE. Wait 1 min and spin again at 12,000 rpm for 20 sec.
11. Run Gel.

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9/~~3~~9/99

Digest:

P1039	60ul
Buffer	7ul
EcoRI	3ue

37°C 2h / 65°C 15min.

9/30/99

1. Run a gel. 70ul.
2. Cut out 7-10kb.
3. Use another kit to extract DNA.

~~Q~~IA quick Gel Extraction kit protocal (another kit)

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weight the gel slice in a tube. Add 3 volumes of Buffer QG to 1 volume of gel (100mg ~ 100ul)
3. Incubate at 50°C for 10min.
4. After the gel slice has dissolved completely, check that the color of mixture is yellow.
5. Add 1 gel volume of isopropanol to the sample and mix.
6. ~~To bind DNA, pipet the sample onto the~~ QIA quick column and apply vacuum. After the sample has passed through the column, ~~switch off~~ vacuum source.

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10/23/97

Run gel. (Southern Blotting)

1. D153 EGoRI Sal I BamHI Xba I 11039EGoR I → in back

2. ~~Rinse # Rinse~~

3. photograph with a ruler laid alongside the gel so that band positions can later be identified on the membrane.

3 Rinse the gel in distilled water and place in a clean glass dish containing ~10 gel volumes of 0.25 M HCl. Shake slowly on a platform shaker for ~~30 min~~ 10 min at room temperature

4. Pour off the HCl and rinse the gel with distilled water. Add ~10 vol denaturation solution and shake as before for 30 min to 1 hr.

5. Pour off the denaturation solution and Rinse the gel with distilled water. Add ~10 vol neutralization solution. Shake as before for 30 min to 1 hr.

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Conjugation: 11/19 20 199

See P10 ;

put the conjugation in

① MGN 617 10^{-1} BHI Km

② 10^{-1} BHI Ap—

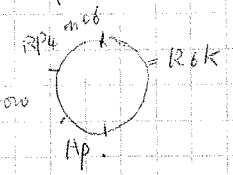
③ 10^{-1} BHI ApKm.

④ MGN 617 PGP 704

10^{-1} BHI Km

10^{-1} BHI Ap → can grow

10^{-1} BHI ApKm

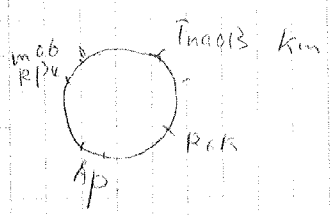


⑤ MGN 617 PLOF Km

10^{-1} BHI Km X2 (100 ul. 20-50 colonies)

10^{-1} BHI Ap

10^{-1} BHI ApKm



100 ul 10^{-5} BHI 11039

10^{-5} LB DAP ApKm → MGN 617

↓

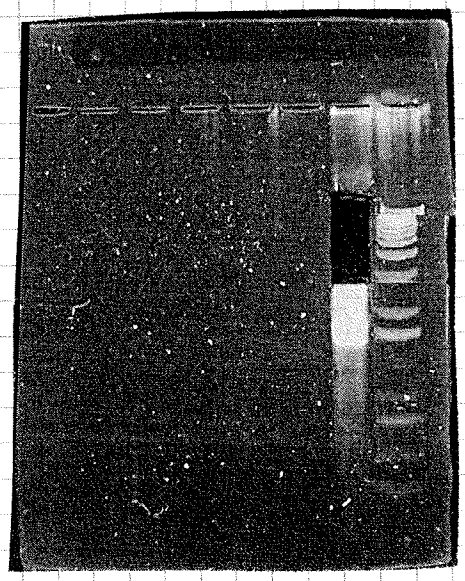
200 ul DAP

11/23/199

Cut out 2-6 Kb, 7-10 Kb, and more than 10 Kb.

Use Qiaquick gel extraction kit to extract DNA.

result:



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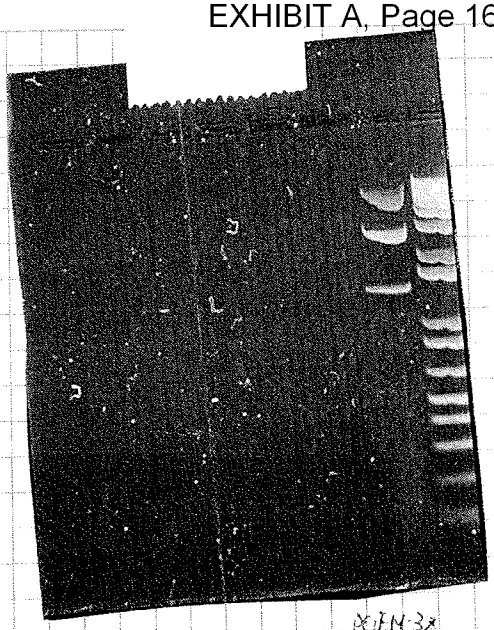

pGEM-3Z
plasmid DNA (1 ul)

The digest is not

The pGEM-3Z has not been digested completely.

So there are three bands. (supercoiled DNA and one strand DNA)

So repeat the digest

11/8/2000

1103 having 1 week long can not grow in BHI broth. but can grow in BHI plate.

Inoculate 1103 and HBN 617. PGN 704. pLO7km again.

Do digest again 2.6 ul DNA (pGEM-3Z)
1 ul EcoRI + 3 ul buffer
over 2h. + 15' 65°C

11/13/2000

Sup 1 ml + 4 ml buffer → 37°C 2h. → 15' 65°C
+ ~~10%~~ 90 ml 100% ECHO + 4 ml 3M NaAc → frazor
+ ~~70% 100 ml~~ 13500 rpm 15' → pour off supernatant →
100 ml 70% ECOH 13500 rpm 10'. → pour off supernant
→ dry → run a gel (1 ml)

Conjugation. LB plate + 200 ul DAP + 100 ul IPTG.
do conjugation

BHI → BHI + 100 ul Ap → BHIAp
BHI + 100 ul km → BHIkm
BHI + 100 ul Ap + 100 ul km → BHIApKm

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2/4/00

~~Run a gel.~~ Digest. 21.5 µl DNA
2.5 µl buffer overnight (B)07
1 µl ECoRI

→ 65°C 15'

Run a gel. cut out ~ 6 kb, 6-10 kb. manchmal 10 (because the extraction DNA always bigger than if is. so. cut out ~~Little~~ was smaller band)

2/7/00

extraute DNA. (use Qiaquick Spin ~~Handbook~~ kit)

2/8/00

Run a gel. but no DNA

improve: Clean DNA befor

2/10/00

☆ Clean DNA

1/10 volume 3M Na acetate.
2 vol 100 % etoh
→20°C ≥ 1h.
centrifuge ≥ 13000 rpm 4°C 15'
Remove supernatant
Add 100 µl 70% etoh
centrifuge ≥ 13.000 rpm 4°C
10 min.
Remove supernatant. air dry ~ 5 min.
Resuspend 30 µl DNAse free H2O
Run a gel → cut out

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2/13/00

1. Run a gel (genomic DNA 11039)
2. Do digest

~~No~~ ghost has a little DNA may because the DNA doesn't ~~beca~~ dissolve well.

3/14/00

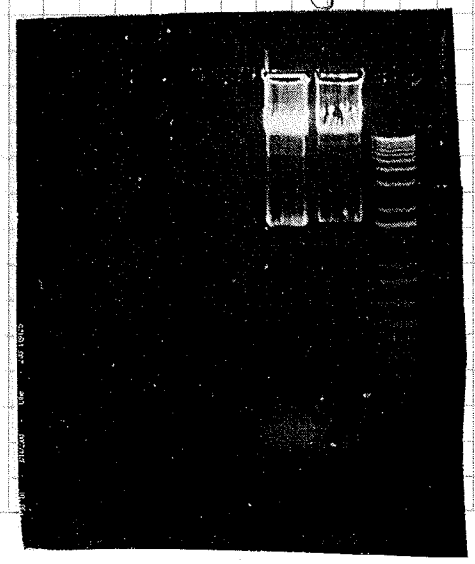
Set up PCR reaction

	①	②	③	④
primer SC 1011	0.8		0.8	0.8
primer SC 1012	0.8		0.8	0.8
dNTP	4	4	0	4
3.3x buffer	6	6	6	6
Mg	2.4	2.4	2.4	2.4
H_2O	6	7.6	10	6
	20ul	20ul	20ul	20ul
higher Layer				
Control DNA	0	0	0	0.5
3.3x buffer	9	9	9	9
Enzyme	1	1	1	1
H_2O	20	20	20	19.5
	30ul			

put 11039 in 50° for 2 hrs (more than) 3/14/00 because a lot of DNA do not dissolve in TE buffer.

Run a gel (11039 genomic DNA.

① Line 1ul DNA)
② Line 3ul DNA.)



Do digest (11039) 75 ul total

62.5 ul DNA
7.5 ul buffer
5ul Enzyme

} 37° overnight

65°C 15'

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in 3 sq rubber maid dishes put 2 pcs chromotograph filter paper (this is too soak up buffers)

1) Denaturation solution - takes very little just enough to soak up filter. 3 min.

2) Neutralization buffer 5 min.

3) 2x SSC 5 min. place it on the paper and dry for 30' cross link on program c3 → put them in plastic.

4113100.

1. preheat the hybridization buffer to 42°c.
2. In a suitable container prewet the blot in 5xSSC. Loosely roll the blot and place inside the tube. Add a small amount of 5xSSC to the tube and "unroll" the blot ensuring no air bubbles are trapped between the membrane and the tube. Don't allow the blot to overlap itself.
3. Pour off the 5xSSC and add the appropriate volume of ~~Hyb~~ hybridization (20ml). buffer.
4. Prehybridize in oven for at least 30 min at 42°c.
5. prepare the labbeled nucleic acid probe as instruction P13.
   - ① Dilute the DNA to be labelled to a concentration of 10 ng/ml using the water supplied.
   - ② Denature 100ng of the DNA sample (10ml) by heating for 5 min in a boiling water bath. (3ul dam + 7 ul H_2O) by adding the
   - ③ immediately cool the DNA on ice for 5 min. Spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.
   - ④ Add an equivalent volume of DNA labelling reagent (10ml) to the cooled DNA. Mix gently but throughly

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5/1/00

prepare sequencing for genomic DNA

Set up sequence program. (Liang sequence)

	Big dye	30-30mer primer (pmoles)	purified gen DNA	H_2O
Roe-Lab 1x reaction	8ul	1ul (30pmol/ul)	11039 6ul	5ul

total 20ul.

p.s. primer. (add 3ul primer to 97ul H_2O)

HAPDAM P2

HAPDAM M2

↓ PCR.

5/2/00 prepare sequencing

1. Column Hydration.

① Remove the top of column, then add 0.8ml of reagent grade water. (invert for a few times. make sure no bubbles) leave the column for at least 2 hrs. at room temp.

2. Removal of Interstitial Fluid.

① remove the top cap first. then remove the end stopper from buttom.

② allow excess column fluid to drain into a wash tube discard this fluid.

③ spin the column and wash tube in centrifuge. at 750g for 2 mins discard it.

3. Sample processing: (it's on another (collection) tube)

① Add 20ul ~~seq PCR~~ sequence(PCR) production into ~~tube~~ column. make sure it's be into the column, but Don't touch the column.

② spin ~~the~~ column at 750g for 2 min. the purified sample will collect in the collection tube

③ Dry the sample in a vacuum centrifuge

4. ① ~~Vorte~~ resuspend sample in 25ul of template reagent in ..

② vortex 30sec } x2
quick spin }

2min denature in 95°C

Chill 5 min.

put 10ul in sequencing tube.

freeze remaining 15ul at -20°C

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H.O sequencing didn't work.

1780 ~~100~~ ul => 1780000 colonies / ml

$$\frac{1.78 \times 10^5}{0.005\ ug} = 3.56 \times 10^7 \text{ / ug DNA}$$

Result: the problem is xgal. the old xgal is not useful for producing blue colonies

Do electroporation again. and inoculate the restreaked bacterials to the LB Broth.

6/13/00

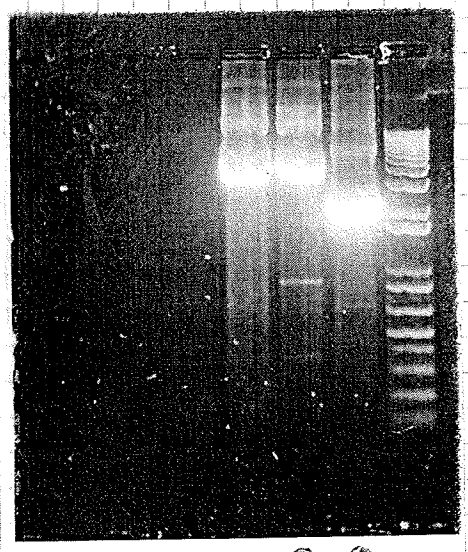
Extract the plasmid DNA (restreaked Bact.)

put the electroporation to refrigiration

Run a gel (plasmid DNA ~~EE~~ ① ② ③)

↓ 2ul + 3ul + 1ul dye

DNA H2O



③ ② ①

② and ③ is defenitely inserted. DO the digest.

20 ul. total

1 ul DNA

2 ul buffer

0.5 ECoRI

16.5 ul H2O

overnight 37° C load 3-4 ul on a gel.

6/14/00

Do ~~electroporation~~. southern hybridization:

1. Run a gel (plasmid DNA ECoRI digested. (20 ul)

load 3-4 ul per well.

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☆ 2×SSC 5 min ×2 at room temp.

5×SSC 0.5% SDS 42°C 30' 摇 oven
[illegible] 50 ml 2.0 ml 10%

2× SSC 用软纸 (Kimwebs) 将膜两面擦.

2× SSC 洗干净 rinse and shake 5 min ×2

7/9/00

Do Ligation 1 ul PGEM-3Z EcoRI + Sap trt.
8 ul insertor (11039 3-5kb extraction)
1 ul buffer
0.5 ul ligase (New England Lab)
16°C overnight

7/10/00 65°C 15'

Do electroporation.

Note: cuvette should be chilled 5 min on ice.
immediately add 1 ml LB medium and transfer to a sterile culture tube.

7/20/00

Run a longer gel for 11039 EcoR I. 5:30 pm. 20V.
1 lane: ladder (2ul)
3 lanes: 11039 EcoRI.
5 ---: ladder (4ul)
It goes very slowly. I should run this gel at 30V.
12:45 end.
1:10 continue
6:10 end.

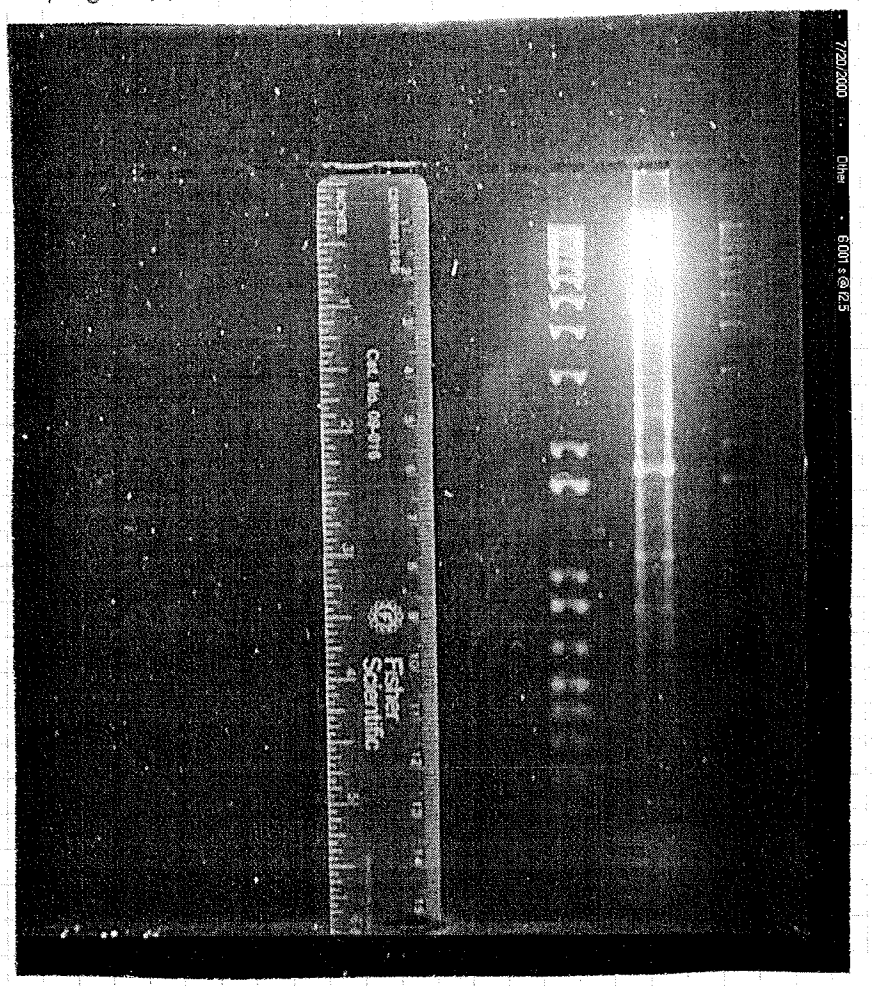
gel size 6 x 15

paper towel 8 x 15

filter paper 3 (~~5~~ 5.5 x 14)

filter paper 5 (8 x 15)

membrane 7 x 14



the part from 6 kb to 8 kb has been seperated. Do Southern hybridization.

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Make hybridization buffer.
100ml buffer
5 g Blotting reagent.
0.6 mol NaCl 2.922 g.

room temp. stir for 1 hr.
then 42°C at least 30'.

8/03/00
Continue Southern hybridization and Sequencing.

8/04/00
Sequencing didn't work again, but Southern hybridization did work.
Set up the PCR reaction to make more 1-10-2 and check the plasmid DNA.

primer dilution
95 ul H2O
2.5 ul HAPolammI → stock for PCR reaction
25 ul ~~~ P1
100 ul.
↘ 1ml.

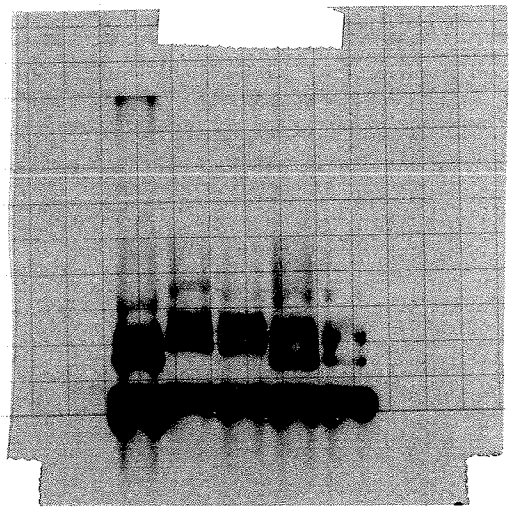
PCR reaction

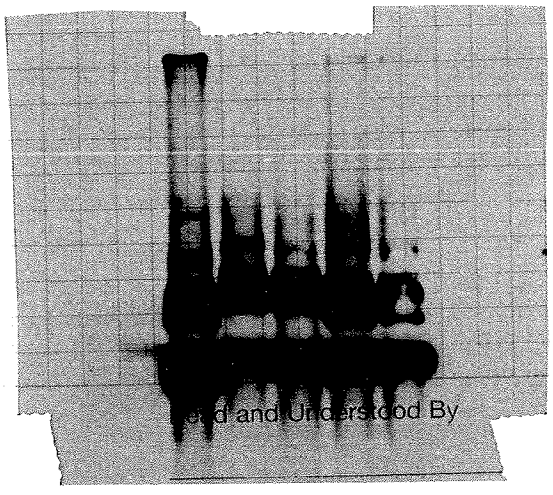
①	1-10-2	0.5
	dNTP	5
	primers	1
	buffer	5
	taq	0.5
	H2O	~~38.5~~ 37.5

②	plasmid	1 ul
	dNTP	5
	primer	1
	buffer	5
	~~0.5~~	0.5
		37.5

③	1-8-1	2
	dNTP	5
	primers	1
	buffer	5
	taq	0.5
	H2O	36.5

④	Control	0
		5
		1
		5
		0.5
		38.5





8/25/00

Run a sequencing reaction

brg dye	8 ul
plasmid	2 ul
primer	2 ul
H_2O	8 ul

Run a gel for genomic DNA (0.5%) in order to comp sepe of ladder

Should keep

~~some~~ a 1.4kb

11039 Hind III

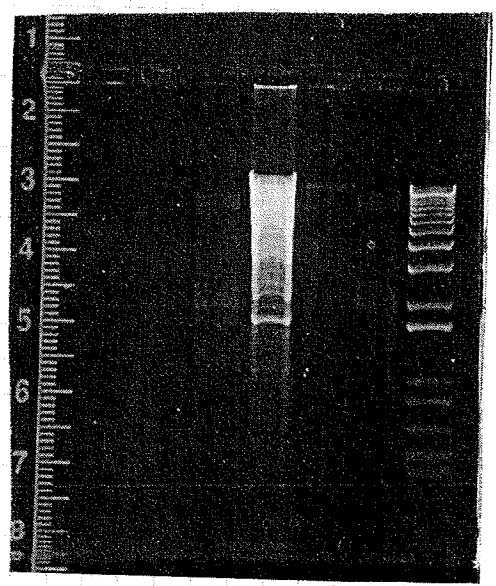
Left for Souther in

8/28/00

Run a gel. 3ul for extracted DNA
3ul for EcoR I 11039 digest (positive control)

① extracted DNA 4-6 kb
② " " 6-9 kb
③ 11039 EcoRI
④ repeat ② → another tube
⑤ repeat ② → another tube

8/29/00



⑤ ④ ③ ② ①

the size of both tubes is bigger then needed. So extract DNA from 3-4 gel size.

8/30/00

Run a gel

10/9/00

Digest clone -a1- a plasmid DNA to get probe

3 ul Digest
3 ul DNA
3 ul buffer
1 ul ~~EtoR7~~ ~~hindIII~~ EcoRI
~~23 ul~~ H2O

30 ul → 2h. 37°C
15' 65°C

cut out from 1.4 kb.

10/10/00

pGEM-3Z hindIII.

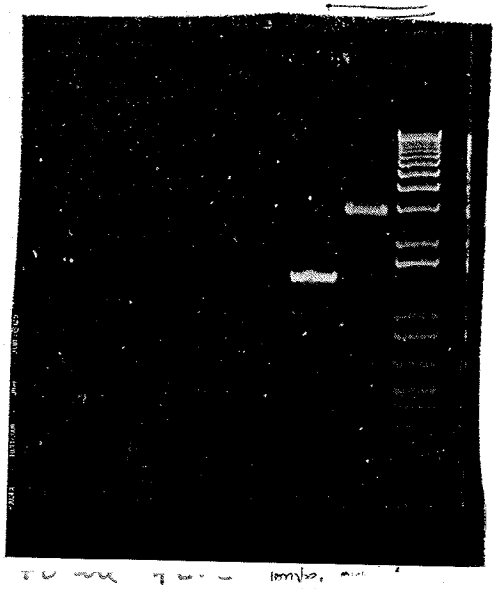
1. Continue plasmid DNA +sap treatment
2. do gel extraction.

10/11/00

Run a gel for gel extraction (for probe) and pGEM-3Z hindIII + sap trt. (both are 5ul)

gel extraction (probe) just need to use 3ul for detect.

I use PBS 1.5ul
Insert 7.5ul
buffer 1ul
Ligase 0.5ul



10/12/00

the electroporation didn't work. try electroporation. 1ul PGEM3Z + [illegible] ampb, amp

↓

LBAp 10^{-2}. 10^{-3}.

to

10/13/00 it did work.

Read and Understood By

Signed

Signed

digest

	①	②	③
DNA	1	1	1
buffer	5	5	5
ECORI	0.5	0.5	0.5
H_2O	43	43	43

50 ul

1/31/01

Do per again

	①	②	③
P multocida A:3	0	1	1
dNTP	5	5	5
primer	1	1	0.5
buffer	5	5	5
New taq	0.5	0.5	0.5
H_2O	38.5	37.5	38.0

50 ul

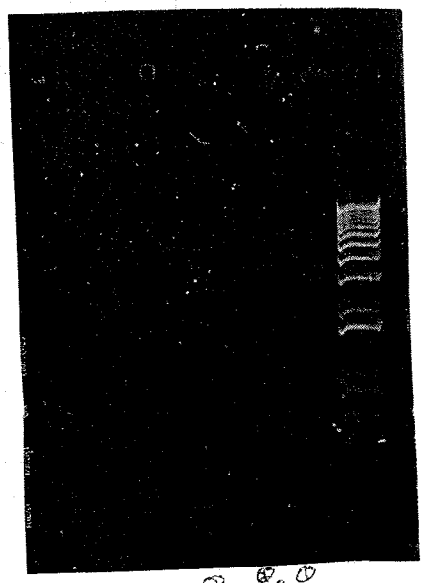
95°C 2min

95°C 30sec. 64°C 1min 72°C 2min 1 cycle

72°C 10min 35 cycles

4°C hold

there're a little DNA on the gel. It's not clear. So Add more genomic DNA to do PCR again



③ 1ul ①

2/01/01

Do PCR again. Add more genomic DNA and increase ~~decrease~~ annealing temperature to 66°C.

	①	②	③	④
P. mul (A:3)	0	3	6	10
dNTP	5	5	5	5
primer	1	1	1	1
buffer	5	5	5	5
Taq	0.5	0.5	0.5	0.5
H_2O	38.5	35.5	32.5	28.5

50 ul

annealing temperature 66°C 1'

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3/5/01

1. Run a gel for H digest.

2. Do a Klenow for clone A ~~EE~~ cla I and T4h III I digest

Klenow: 20 ul digest (the concentrat of my DNA is high enough)

0.8 ul dNTPs (200 uM of each in working solut)

1 ul 0.1 mg/ml BSA (I skip this step)

0.5 ul Klenow

I didn't add buffer because the Multi Core (buffer) is compatible for Klenow

incubate @ 37°c for 30 min. H I @ 75°c for 10 min.

(I lost this Klenow product)

3/6/01

Do digest for B. C. E. T. G.H. and Rnd a gel.

for it.

I select B. C. G to run a seq. reacti again.

And to make sure which one is right.

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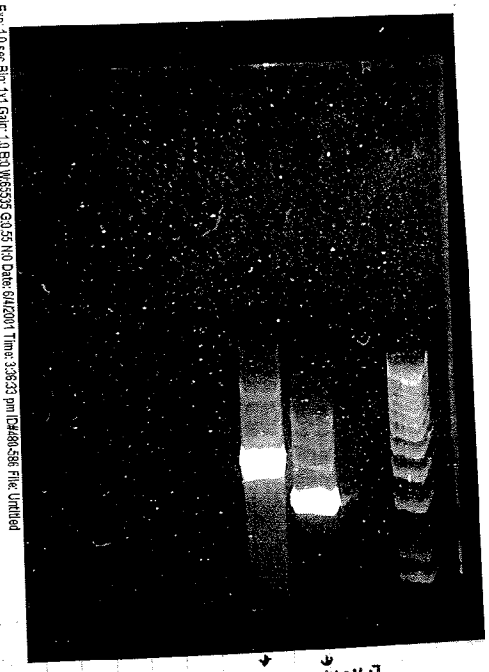


PPS88 ↗ MGN617

615101.

Do the digest for PPS88 (Xba I + Kpn I)

5 ul DNA

12 ul H_2O

(multi wire) buffer 2 ul

0.5 ul XbaI

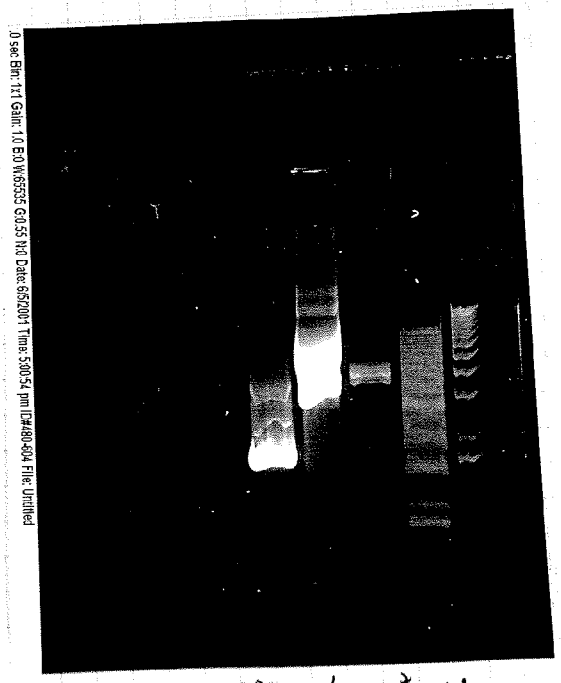
0.5 ul KpnI

2h 37°C

inactivate 65°C 15'

Run a gel for digestion, genomic DNA ↓ PCLPm2. (from clone A) and pbluescript plasmid

all the samples → 1 ul



PPS 88 clone A ↗ MGN. plasmid

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do sequencing react. for number 5

big aye 8 ul
plasmid 2 ul
primer T3 2 ul
water 8 ul

Run a gel for ① ③ ⑤

Sample	Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
pbluescript and dam ⑤	dsDNA	3.631	5.554	3.175	0	1.74929	0.2777 ug/uL	1	50	174.93

↓ number 5.

6/29/01

Sample	Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
pLS88	dsDNA	0.577	1.176	0.606	0	1.94059	0.0588 ug/uL	1	50	194.06

8 ul PLS 88
XbaI / BamHI 0.5 ul
buffer (multicore) 1 ul
water 0.5 ul

8 ul PLS 88
Xba I 0.5 ul
BamHI 0.5 ul
multicore 1 ul

7/3/01

PCR:

				control
Template	pCLPM3	0.5 ul	1 ul	0
dNTP		5 ul	5 ul	5 ul
primer		1 ul	1 ul	1 ul
buffer		5 ul	5 ul	5 ul
Taq		0.5 ul	0.5 ul	0.5 ul
H2O		38 ul	37.5 ul	38.5 ul

primer dilut.

95 ul	H2O
2.5 ul	T7P1
2.5 ul	CLdam M20

568 8362-118 Jun 12 2001
GENOSYS
T7P1
5'-GGATCCTGCGTTATCCCC TGATT
1361.6µg 40.4OD 195.0nmol
Tm=69.7°C 33.7µg/OD MW=6981

568 8362-119 Jun 12 2001
GENOSYS
CLDamM20
5'-TCTAGATGTTGCCAATGC CAGTGTA
579.6µg 18.3OD 75.5nmol
Tm=69.2°C 31.7µg/OD MW=7672

GENOSYS
568

Signed

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Signed

8/15/01 Cont.

PCR4 XL1B electroporation...

Blotted plates with Hybond membrane

followed protocol for colony Hybridization

Cross linked membrane - stored at -20C ON

8/16/01

Colony Hybridization PCR4 XL1B

made probe using 3ul PCR-4 + 7ul H2O in ECL Kit

preheat Hybridization buffer to 42°C

wet Screen mesh + Membranes

Roll + put in hybridization tube

pre hybridize with SSC 5X for 1 Hour

use 30mls Hybridization Buffer

probe Boil 5"

Cool on ice 5"

10 ml labelling reagent

10 ml gluteraldehyde

10" 37°C

add 1ml hybridization buffer from tube - add buffer + probe to tube 42°C ON

Friday 8/17/01

Colony Hybridization Cont

preheat 1° wash buffer to 42°C

discard Hybridization buffer ~~Add SX SSC~~

Add 50ml 5X SSC return to Oven 5 min

discard 5X SSC add 1/3 vol of tube w/ 1° Wash But

Return to Oven for 20min

Wash again in 1° wash buff for 10 min 2X

remove blots from tube place in glass container

Cover w/2X SSC

Shake @ RT for 5 min X 2

Dectection

8ml/membrane 13mls #1 13mls #2 3membranes

Incubate 1 min

place on Saran

Read and Understood By

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Signed

Notebook Number: _____

Date: _____

9/19/01 Cont.

Culture reached 260 KU after 4 hrs incubation at 37°C

(2) Put cultures into 8534 tube

(3) Incubate on ice 15"

(4) Cf 4°C 5000 x g (6500 rpm) 15"

(5) Wash 2X w/ 1mM Hepes buffer (pH 7.0)

(6) resuspend pellet in 10% glycerol to 1/2 their Original Vol = 2 mls

(7) Cf 5000 x g 10"

(8) resuspend in remaining glycerol solution after supernatant is decanted

(9) Flash freeze in EtOH + dry ice bath

(10) Freeze at -80°C

P. mult 11039 is highly encapsulated + forms a very soft pellet I lost the pellet in the 1st wash step, so more cells will have to be prepared

Electroporate pLS88 into P. mult 1069

1 ul pLS88 plasmid prep (in plasmid box spot G9)

40 ul 1069 comp cells

2.5 KV
200 Ω
25 uF
Tc = 5.12 msec

Incubate 37°C 1 hr in LB

RESULTS

10^{+1}	134 colonies
10^{0}	2
10^{-1}	0

plate 10^{0}, 10^{-1}, 10^{+1} 100 ul on LB strep 37°C

10^{+1} = after plating 10^{0} + diluting 10^{-1}, Ct culture + resuspend pellet in 100 ul media - plate 100 ul

9/25/01 Tuesday

Pass colony from R6Ei2 #4 blot - do a plasmid prep

Electroporate pLS88 into P. mult 11039

1 ul pLS88 plasmid prep (in plasmid box spot G9)

40 ul 11039 comp cells (flash frozen in liq N2)

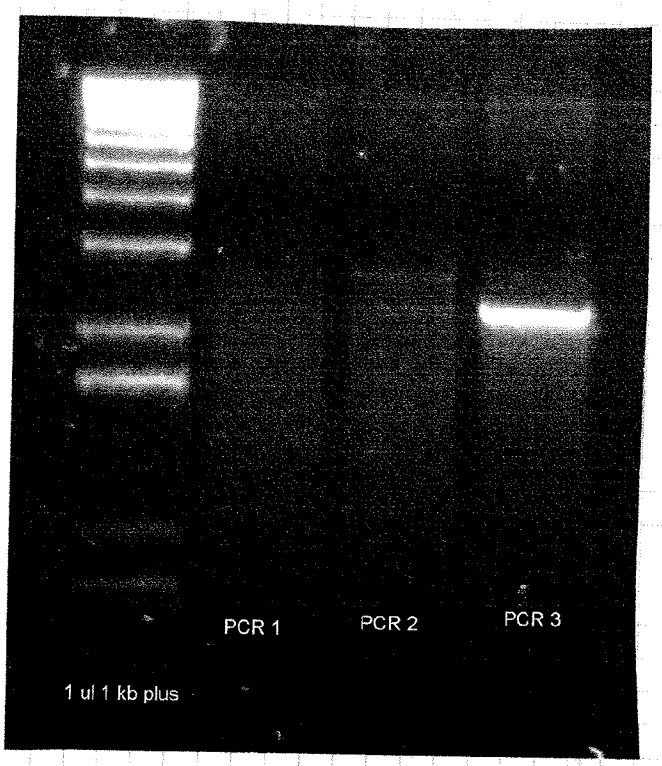
2.5 KV
200 Ω
25 uF
Tc = 5.16 msec

RESULTS

10^{+1} 80
10^{0} 13
10^{-1} 1

Incubate 37C 1 hr LB

Plate 10^{0}, 10^{-1}, 10^{+1} 100ul on LB strep 37°C



See pg 35 for PCR rx setup

Annealing temp = 48°C

Extension time = 30 sec

pcr rx did not work again

0.7% agarose 3.5 ul gel star
5 ul of PCR Rx loaded

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Signed

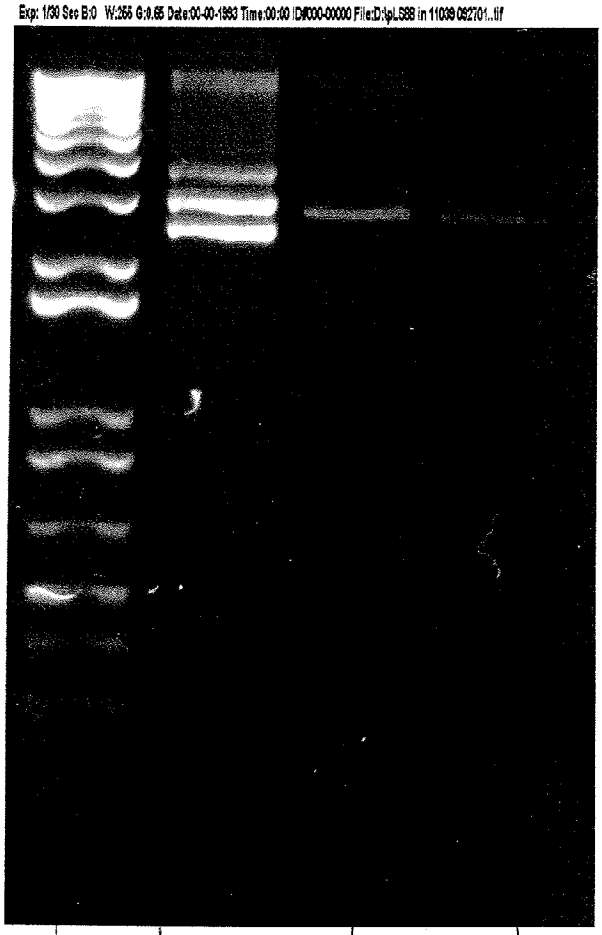
Notebook Number: _____

Date: _____

9/26/01 Wednesday - Gone to Memphis
ML started 5ml culture of ~~pMBXpelE1Z~~ R6E1Z Ed 9 +

9/27/01 Thursday

Did a Qia Spin mini prep of R6E1Z
Eluted in 30ml EB

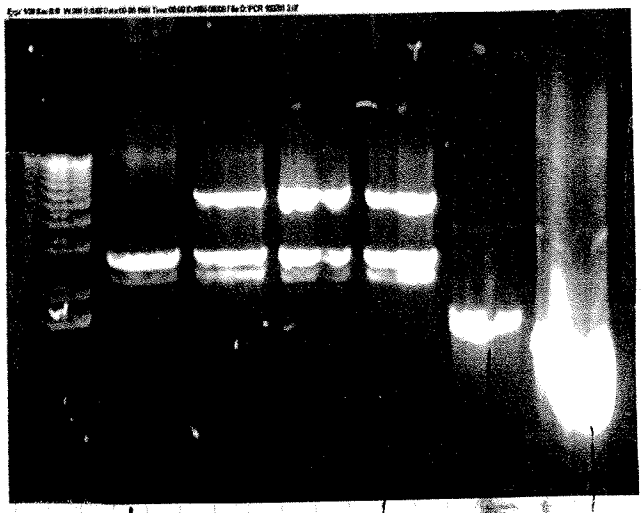


R6E1Z looks good
Pass to a new plate
freeze next week

PLS88 in 11039 looks good -
it work - we were able to electroporate into P.multocida Comp cells

PCR pCLpm3 T7P1 + CLdamM20 092801			
Sample #	1	2	3
pCLpm3 8/29/01 BYR	0.5	1	0
dNTPs	5	5	5
T7P1 + CLdamM20	1	1	1
Taq	0.5	0.5	0.5
Taq Buffer	5	5	5
Water	38	37.5	38.
TOTAL Rx amt.	50	50	
Annealing temp	67.5 C		

10/2/01



PCLpm3
1ul

See pg 41 PCR Set up

10ul cut out of gel = 0.4177 gm
put in Ref over night

10/3/01

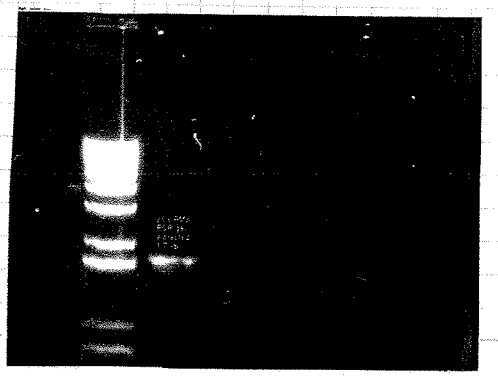
Elute DNA from gel with Qiagen gel extraction Kit

~~cut~~ cut gel fragment in 1/2 and put in 2 tubes
Added 600ul / tube of QG
Added 200ul Isopropanol

eluted each column w/ 30ul EB into same tube = 60ul total

Called pCLpm3 PCR

Loaded 5ul of pclpm3 PCR on 0.7% agarose gel 3.5ul gelstar
100 Volts



Read and Understood By

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Signed

Notebook Number: _____ Exhibit A Page 36

Date: _____

10/3/01

Electroporated 40 ul P. multocida 11039 Comp Cells w/no DNA

Recover 1 hr in LB broth 37 C

plate 100 ul 10^0, 10^{-1}, 10^{+1} on LB grow at 37 C ON

Should have plated on LB strep – threw plates away

2.5 KV
25 uF
200 Ω
Tm = 5.16 msec

Before Digesting pCLpm3 PCR 1.7 Kb frag we need more PCR product b/c XbaI has to be deactivated w/ EDTA (heat does not work) + then the DNA is ppct out. DNA is lost in ppct so I need to amplify my PCR product

PCR pCLpm3 1.7 kb T7P1 + CLdamM20 10/3/01			
Sample #	1	2	3
pCLpm3 PCR 1.7 kb	0.5	1	0
dNTPs 1mM each	5	5	5
T7P1 + CLdamM20	1	1	1
Taq (7/14/01)	0.5	0.5	0.5
Taq Buffer	5	5	5
Water	38	37.5	38.5
TOTAL Rx amt.	50	50	50
Annealing temp	67.5 C		
extension time	30 sec		

run 0.5 ul pcr rxs on gel

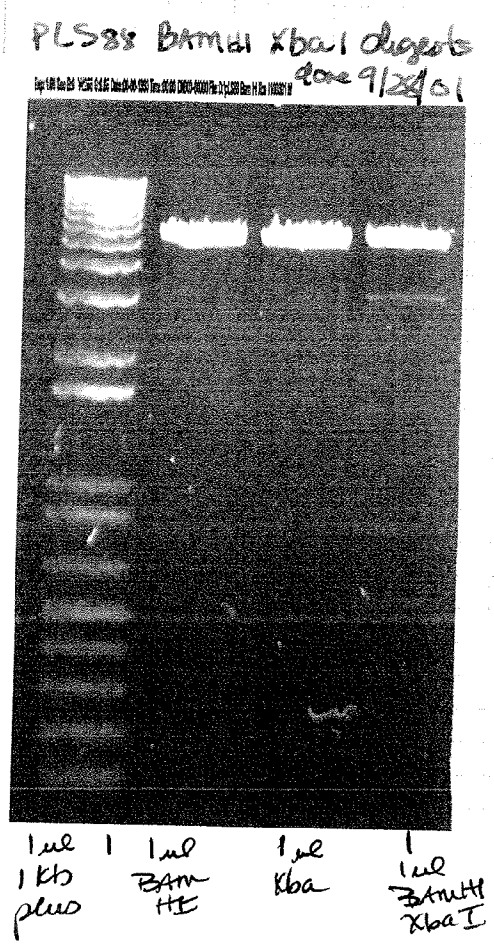
Read and Understood By

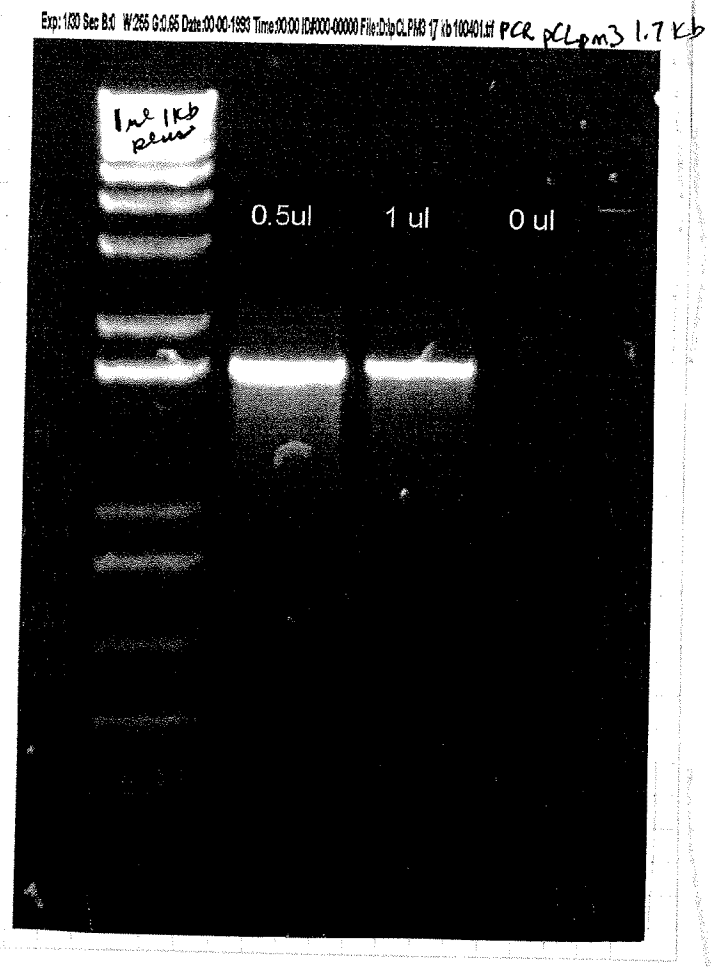
pLS88 digest BAMHH/Xba I

10ul	pLS88 plasmid prep 9/19/01
1ul	Xba I
1ul	Bam HI
2ul	Multicore Buffer 10X
6ul	H2O
20ul	

Digest 37 C 4 hrs Start 10:00am

Run 0.5ul of pCLpm3 1.7Kb pcr Rx run 10/3/01 on 0.7% agarose gel 3.5ul gel star





Read and Understood By

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10/4/01

Digest pCLPM3 1.7Kb frag PCR Rx 1 (from 10/3/01)

10 ul	DNA	
1 ul	XbaI	37 C
1 ul	BamHI	
2 ul	MultiCore 10X Buffer	4 hrs
6 ul	H2O	
20 ul		

To pLS88 & pCLPM3 1.7Kb BamHI XbaI digests

Add 0.5ul 0.5M EDTA to deactivate XbaI
Add 2 ul 3M NaAcetate (1/10 vol)
44 ul ETOH (2 vols)

freeze -20 overnight to ppct DNA.

Start 2 1L BHIAp cultures of RbEi2 for LPS prep
Add 2.5mls of ON culture per Liter
add 500ul 200mg/ml Ap = 100ug/ml final vol
1/2 normal conc. used
grow ON 26°C Shaking

10/5/01

Cf. RbEi2 cultures in 250ml bottles in GSA rotor
6000rpm for 15min at 4°C
freeze pellets at -20 C.
Do LPS prep next wk

Read and Understood By

10/5/01

Cf. ppt DNA pLS88 + pCL PM3 1.7Kb resuspend in 10 ul TE. put at 4C to go into suspension

10/8/01

run 1ul pLS88 + pclpm3 1.7Kb on gel
✓ on gene spec first

Sample	Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
pCL PM 3 1.7 kb	dsDNA	0.655	1.536	0.818	0	1.87775	0.0768 ug/uL	1	50	104.32
	dsDNA	0.707	1.592	0.85	0	1.87294	0.0796 ug/uL	1	50	104.05
pLS88 Bam HI Xba I	dsDNA	1.565	3.982	2.369	0	1.68088	0.1991 ug/uL	1	50	93.38
	dsDNA	1.987	4.493	2.85	0	1.57649	0.22465 ug/uL	1	50	87.58

pCl pm 3 76.8 ng/ul pLS88 211.9 ng/ul

Ligation

pLS88 4.5 Kb
pCL PM 3 1.5 Kb

pLS88	1ul	200ng
pCL Pm3	7.5ul	570ng
Ligase Buff	1 ul	
Ligase	0.5ul	
	10ul	

14°C overnight

10/9/01 Tuesday

Heat inactivate ligation 65°C 15'

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Signed

PCR 93146 Ei OPS primers 10/17/01				
Sample #	1	2	3	4
93146 genomic prep 7/28/99	2	2	2	2
dNTPs 2.5mM	10	10	10	10
Ei OPS PCR U 2 + Ei OPS PCR R 2	1.2	1.2	1.2	1.2
EXL polymerase	1	1	1	1
DMSO	0	0.5	1	1.5
Stabilizing Soln	1	1	1	1
10 X Buffer	5	5	5	5
Water	29.8	29.3	28.8	28.3
TOTAL Rx amt.	50	50	50	50
Use PCR EXL Pcr Program				
Annealing Temp	63			
PCR EXL primer				

Ran out of EXL polymerase after 1st Rx.
Ran 1st Rx ordered more EXL poly

Rxs 2-4 are in green RCR box -20 chest freezer

gel picture pg 54

Make 2 5ml LBAP cultures of pFPV25 in DH10Bs
1 - plasmid prep
1 - freeze

Make 5ml cultures of pLS88 isolates pos + Adj on LBstrep
positive colony from hybridization → adjacent to positive colony

Read and Understood By

Signed

Signed

10/18/01

Started cultures in wrong Antibiotics
Started pFPV25 in LB strep · pLs88 in LBAp Opps -

Start new 5 ml cultures
pFPV25 in LB Ap
pLs88 in LB Strep

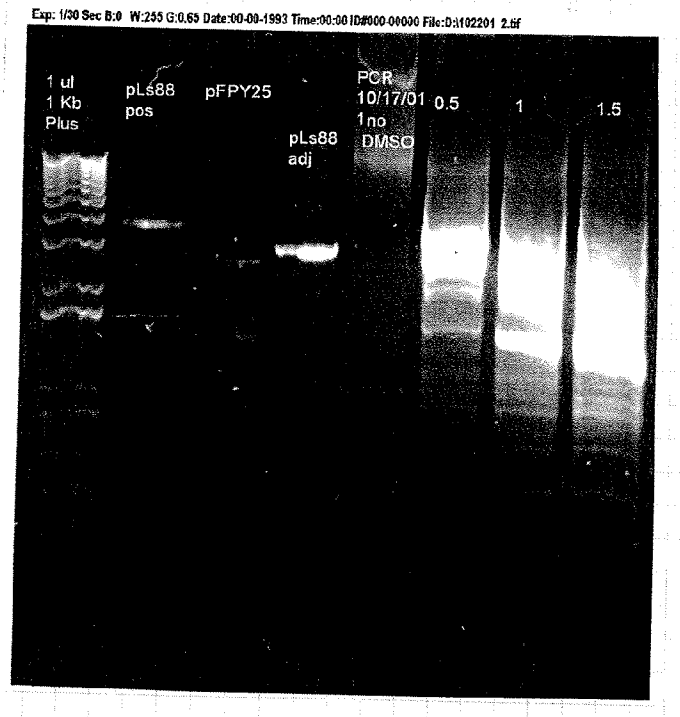
10/19/01

Froze 1 pFPV25 culture (Ask BYL where it is)

Spin down other pFPV25 culture + both pLs88 cultures (pos + adj) - freeze pellets for plasmid preps

10/22/01

made Qiaspin plasmid preps of
PFPV25, pLs88 positive, pLs88 adjacent



Loaded 1ul of
pLs88 pos
pLs88 adj
pFPV25

Loaded 5ul of
pcr producted

pass both pLs88 colonis
to A new LB strep plate

Read and Understood By

10/23/01

Digest pLS88 pos + Adj plasmidpreps (10/22/01)

Cut out insert

	pLS88 pos	pLS88 Adj
BAM HI	1 ul →	1 ul →
Xba I	1 ul →	1 ul →
multicore	2 ul →	2 ul →
H_2O	12 ul →	12 ul →
	20 ul →	20 ul →

load 10 ul on gel →

BAMHI / Xba I cuts the insert out

Linearize

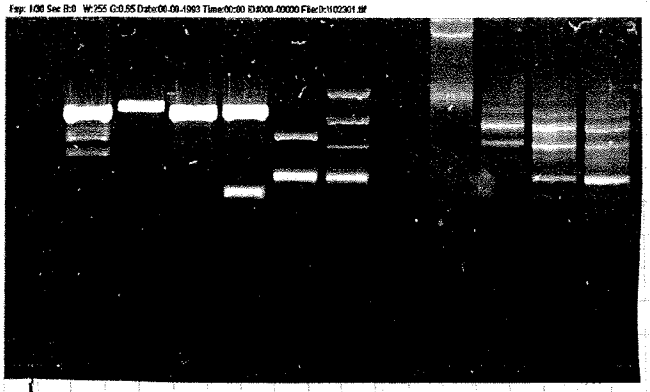
	pLS88 pos	pLS88 Adj
EcoRV	1 ul	1 ul
Buff D	1 ul	1 ul
H_2O	6 ul	7 ul
	10 ul	10 ul

load 10 ul on gel →

Linearize DNA

	pFPV25	pFPV25
EcoRV	1 ul	
Buff D	1 ul	
H_2O	3 ul	
pst		1 ul
Buff H		1 ul
H_2O		3 ul
	10 ul	10 ul

load 10 ul on gel →



1 Kb plus did not show up

1) pLS88 adj BamHI/Xba I 10 ul + 2 ul 6X Dye
2) pLS88 pos EcoRV
3) pLS88 Adj EcoRV
4) pLS88 pos BAMHI/Xba I
5) pFPV25 pst I
6) pFPV25 EcoRV
7) PCR 10/17/01 No DMSO 2 ul DNA + 1 ul 6X dye + 3 ul H_2O
8) 0.5 ul
9) 1 ul
10) 1.5 ul

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Signed

Signed

10/24/01

PCR pMBEi1 Ei OPS primers 10/24/01				
Sample #	1	2		
From 9/7/00 in MBEi1 pMBEi1 DNA Box	0.5	0.5		
dNTPs 2.5mM	10	10		
Ei OPS PCR U 2 + Ei OPS PCR R 2	1.2	1.2		
EXL polymerase	1	1		
DMSO	0	0.5		
Stabilizing Soln	1	1		
10 X Buffer	5	5		
Water	31.3	30.8		
TOTAL Rx amt.	50	50		
Use PCR EXL Pcr Program				
Annealing Temp	63			
PCR EXL primer				

pLS88 positive gene spec

Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
dsDNA	3.461	5.406	4.733	0	1.14219	0.2703 ug/uL	1	50	63.46
dsDNA	0.046	1.798	1.112	0	1.61691	0.0899 ug/uL	1	50	89.83
dsDNA	0.018	1.781	1.091	0	1.63245	0.08905 ug/uL	1	50	90.69
dsDNA	0.031	1.779	1.083	0	1.64266	0.08895 ug/uL	1	50	91.26
dsDNA	0.3	2.076	1.421	0	1.46094	0.1038 ug/uL	1	50	81.16
dsDNA	0.732	2.531	1.855	0	1.36442	0.12655 ug/uL	1	50	75.80
dsDNA	0.538	2.393	1.707	0	1.40187	0.11965 ug/uL	1	50	77.88
dsDNA	0.37	2.2	1.522	0	1.44547	0.11 ug/uL	1	50	80.30

plss88 plasmid prep made 10/24/01 Conc = 124.8 ng/ul

Seq pls88 w/ CLPM1-PL 3.2pmol → p.29 bkV + w/ T3 3.2pmol → pg 27 bkV

pLs88 4ul
TRR 8ul
primer 2ul
H2O 6ul
20ul

#1 CLPM1-PL
#2 T3

Sequence should contain DAM gene sequence
CLPM1-PL primer sequence data is trashy - but
I blasted a segment of it and it is the DAM gene
sequence

Redigest pFPV25 5ul
Buff H 1ul
H2O 3ul
EcoRI 1ul
[illegible]
10ul digest

Pst I 1ul

pFPlas 5ul
EcoRI 1ul
pst I 1ul
Buff H 2ul
H2O 10ul
20ul

Read and Understood By

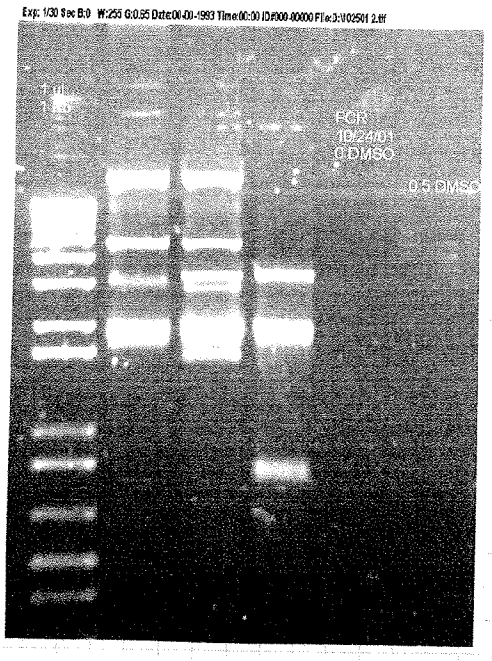
Signed

Signed

Notebook Number: _____

Date: _____

10/25/01



0.7% agarose 3.5ml gl star

1) 1 µl 1 Kb plus
   + 4 µl H_2O
   + 1 µl 6x dye
2) pFPV25- EcoRI 10 µl + 2 µl
3) pFPV25 pst1 10 µl
4) pFPV25 EcoR1/pst1 10 µl
5) PCR pMBE.1 0.5 µl No DMSO
6) " " " 0.5 DMSO

   + 1 µl 6X dye
   3.5 µl H_2O

Freeze pls88 pos [redacted]

Start 5ml LB strep

Electroporate pls88 pos into P.mult 11039 comp cells

2.5 KV
200 Ω
2.5 µF
Tc = 5.08ms

40 µl P.mult (A11 D4 9/21/01)
1 µl pls88
Recover 1 hr LB strep

plate 10^{+1} on LB strep

Incubate 37 °C ON

10/26/01

Make Qiaspin plasmid prep of pFPV25 in DH10βs
Elute 30ul EB
Digest w/ EcoR1 / Pst 1 (same as yesterday)
digest 5 hrs

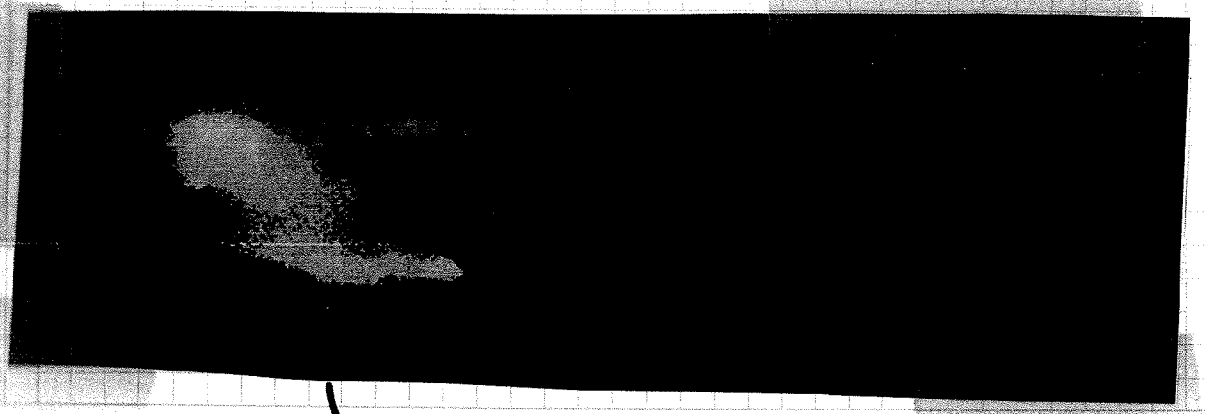
run on gel w/ undigested plasmid

Froze PLs88 pos now called pLs88 DAM
it is in XL1B MRF'
A11 D5-9 LBstrep + 20% glycerol

Put plate from Electroporation in ref over wkend - Monday pick 4 big colonies + plate on LB strep

Started BH1 Ap plate cultures of 93146 WT Lux + 93146 Rif Lux

Imaged fish immersion + ip injected w/ 93146 WT Lux on Night Owl.



Luminescent area

Read and Understood By

Signed

Signed

pLS88 positive gene spec

Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor	Purity
dsDNA	3.461	5.406	4.733	0	1.14219	0.2703 ug/uL	1	50	63.46
dsDNA	0.046	1.798	1.112	0	1.61691	0.0899 ug/uL	1	50	89,83
dsDNA	0.018	1.781	1.091	0	1.63245	0.08905 ug/uL	1	50	90.69
dsDNA	0.031	1.779	1.083	0	1.64266	0.08895 ug/uL	1	50	91.26
dsDNA	0.3	2.076	1.421	0	1.46094	0.1038 ug/uL	1	50	81.16
dsDNA	0.732	2.531	1.855	0	1.36442	0.12655 ug/uL	1	50	75.80
dsDNA	0.538	2.393	1.707	0	1.40187	0.11965 ug/uL	1	50	77.88
dsDNA	0.37	2.2	1.522	0	1.44547	0.11 ug/uL	1	50	80.30

plss88 plasmid prep made 10/24/01 Conc = 124.8 ng/ul

Seq pls88 w/ CLPM1-PL 3.2pmol → p.29 bkV + w/ T3 3.2pmol → pg 27 bkV

pLs88 4ul
TRR 8ul
primer 2ul
H2O 6ul
20ul

#1 CLPM1-PL
#2 T3

Sequence should contain DAM gene sequence
CLPM1-PL primer sequence data is trashy - but
I blasted a segment of it and it is the DAM gene
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Redigest pFPV25 5ul
Buff H 1ul
H2O 3ul
EcoRI 1ul
[illegible]
10ul digest

Pst I 1ul

pFPV25 5ul
EcoRI 1ul
Pst I 1ul
Buff H 2ul
H2O 10ul
20ul

Read and Understood By

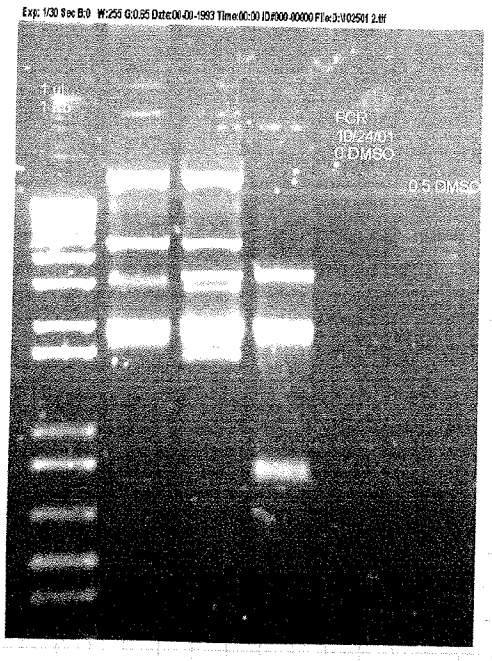
Signed

Signed

Notebook Number: _____

Date: _____

10/25/01



0.7% agarose 3.5ul gl star

1) 1ul 1 Kb plus
   + 4ul H_2O
   + 1ul 6x dye
2) pFPV25- EcoRI 10ul + 2ul
3) pFPV25 pst1 10ul
4) pFPV25 EcoR1/pst1 10ul
5) PCR pMBE.1 0.5ul No DM
6) " " " 0.5 DMSO

   + 1ul 6X dye
   3.5ul H_2O

Freeze pls88 pos [redacted]
Start 5ml LB strep

Electroporate pls88 pos into P.mult 11039 comp cells

2.5 KV
200 Ω
2.5 uF
Tc= 5.08ms

40ul P.mult (A11 D4 9/21/01)
1 ul pls88
Recover 1 hr LB strep

plate 10^{+1} on LB strep
Incubate 37°C ON

10/26/01

Make Qiaspin plasmid prep of pFPV25 in DH10βs
Elute 30ul EB
Digest w/ EcoR1 / Pst 1 (same as yesterday)
digest 5 hrs

run on gel w/ undigested plasmid

Froze PLs88 pos now called pLs88 DAM
it is in XL1B MRF'
A11 D5-9 LBstrep + 20% glycerol

Put plate from Electroporation in ref over wkend - Monday pick 4 big colonies + plate on LB strep

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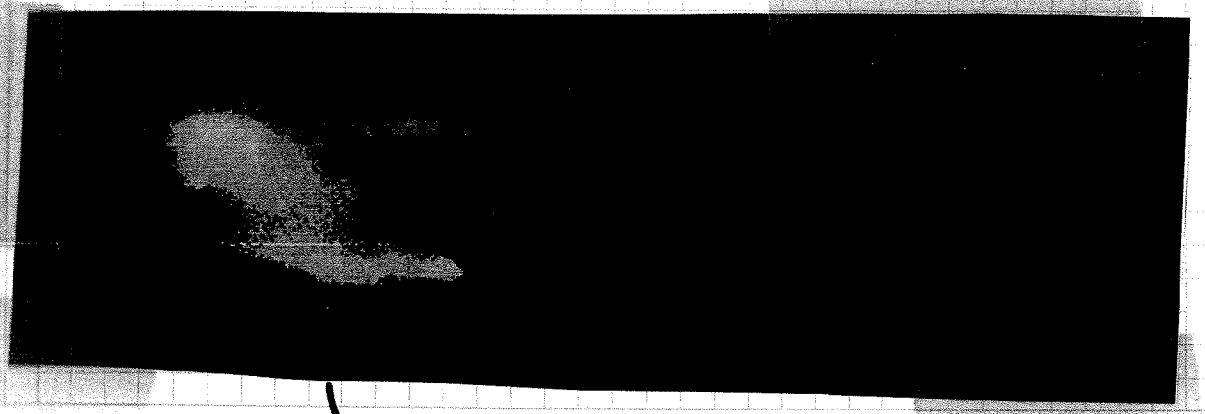


Luminescent area

Read and Understood By

Signed

Signed

Notebook Number: _____

Date: _____

60 10/31/01 Pick 4 colonies from P. mult 11039 pLS88 electroporation. grow on LB strep

11/5/01

pour 12%/4% SDS-PAGE gel
gel stayed in buffer in 4°C ON

Start 5ml LB strep cultures of P. mult 11039 pLS88 From 10/31

11/6/01

Samples to run on gel

LPS from R6Ei2 dilute - 1/1 in sample Buffer

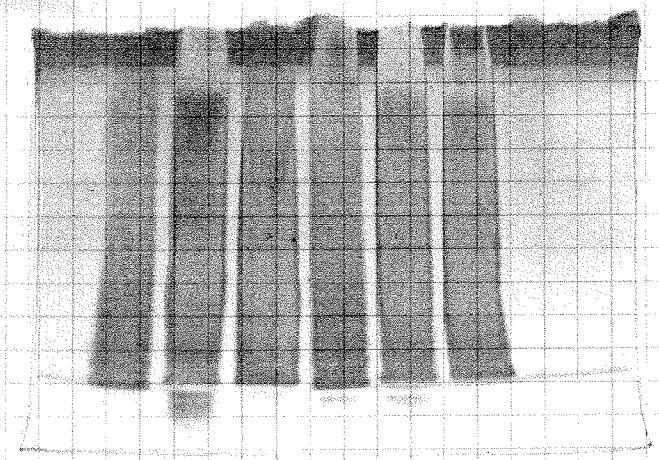
and take 10ul (1mg/ml) + 15ul H2O + 25ul Buffer = 10ug

LPS Salmonella ~~typh~~ 1/1 of 2mg/ml in H2O 1/1 in Buffer

Lane 1 Sal typ
2 R6Ei2 1/1 } Not Denatured
3 R6Ei2 10ug
4 Sal typ
5 R6Ei2 1/1 } Denatured
6 R6Ei2 10ug

loaded 10ul / well Ran at 100volts

Silver stain for LPS



Read and Understood By

Made Qiaspin plasmid preps of P.mult 11039 pLSS8 4 colonies

11/7/01 Run 12%/4% SDS PAGE gel
Same as 11/6/01

1) Sal. typ 1/1 of 2mg/ml in H_2O 1/1 in Buffer - Load 10 ul
2) R_6E_{12} 1/1 in Buffer
3) R_6E_{12} 10ug
4) WT 93146 LPS 10ug

Electrophoresed at 100Volts
tranfered to Nitrocellulose 100 volts 1 hr
Blocked Membrane overnight 5% NFM in PBS
Our Ed 9 was contaiminated - so was Bobbies

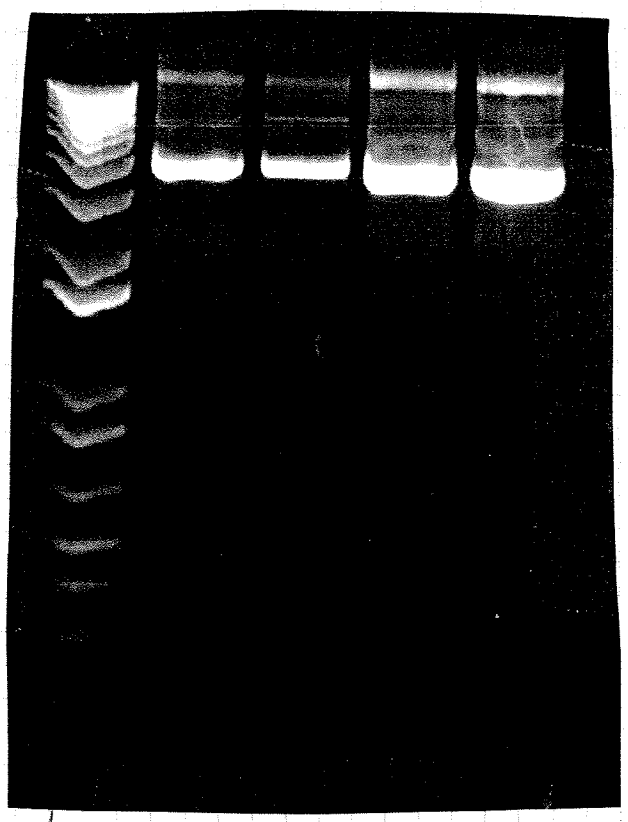
DH10Bs pFPV25 frozen -80C All F1-5
tube #1 used to start a 5ml LBap culture
& put on LBap plate

P.mult 11039 pLSS8 #1 Start 5ml LB strep culture to freeze → see gel pic Next page

Read and Understood By

Signed

Signed



p. mut 11039
Dan gene pks 88
plasmid preps
from 11/6/01

1 ul
1Kb plus

11/8/01

Wester Blot R6E12

There is No Ed 9 to use as 1°Ab so

1° used α Ed ict 4383 polyclonal CF serum 1/1600
+ α Ed ict 93146 wt polyclonal CF serum 100ul added
to 1/1600 dilution

Incubate RT 1 hr shaking
Wash 3X PBS TW20

2° 9E1 1/4 1 hr RT shaking

Wash 3X PBS TW

3° goat α mouse lg AP 1/1500 1 hr RT Shaking
Develope BCIP/NBT 20 mes

It did not work
will have to repeat
when Bobbie has more
Ed 9

1 2 3 4

Frozg pasturella multocida 11039 pls88 (DAM gene)
LB strep + 20% glycerol
-80 C Box A11 G1-5

Digest pls88 p.mult 11039 #1

BamHI/XbaI
DNA 4ul
BamHI 1ul
XbaI 1ul
multicore 2ul
H2O 12ul
20ul

BAM HI
DNA 4ul
BamHI 1ul
multicore 1ul
H2O 4ul

Digest 37°C
for 2 hrs Approx

run on gel tomorrow

Plasmid prep pFPV25 in DH10βa
Eluted in 30 ml Set next pcg for gel prc

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Signed

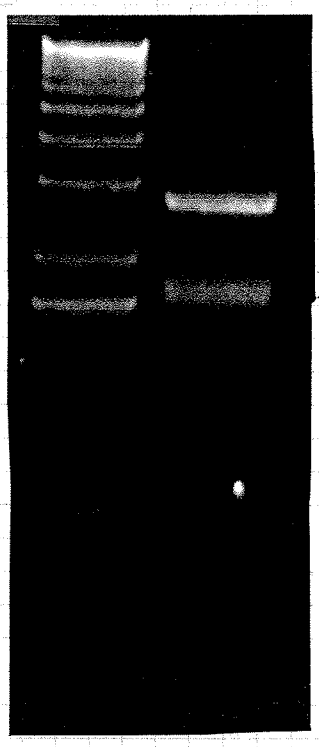
Signed

64

Notebook Number: _____

Date: _____

11/8/01



pFPV25

— do not know what this band is

next Run angel and extract top band & electroporate into XL1B

93146 Lux recovered from fish immersion infected

Bacteria was cultured 4 days post immersion

Lux plasmid is still stable in vivo

Exp: n/a Bin: 1x1 Gain: 1.0 B:0 W:65535 G:0.55 N:0 Date: 11/1/2001 Time: 11:14:39 am ID#0-0 File: Untitled

Neg Controls 14 + 15

22 : 93146 Lux

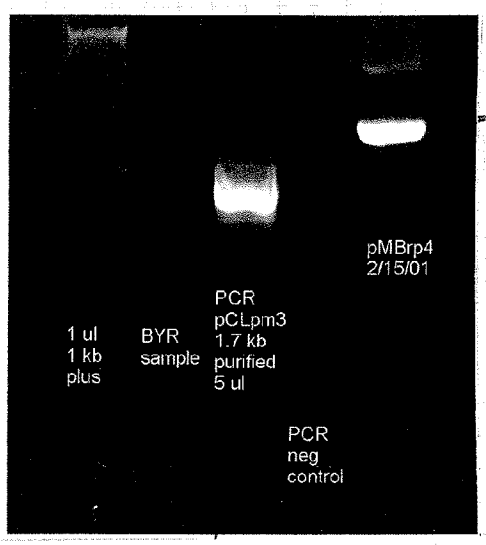
Read and Understood By

PCR pCLpm3 1.7 kb T7P1 + CLdamM20 purified 01/16/02

Sample #	1	2	
pCLpm3 PCR 1.7 kb purified	0.5	0	
dNTPs 1mM each	5	5	
T7P1 + CLdamM20	1	1	
Taq (7/16/01)	0.5	0.5	
Taq Buffer	5	5	
Water	38	38.5	
TOTAL Rx amt.	50	50	
Annealing temp	67.5 C	program #1 under mark	
extension time	30 sec		

1/18/02

run 5ul on gel of PCR Rx 5ul DNA
1ul 6x Dye



measured pMBrp4 on gene spec - got a low reading of 11.8ng/ul
So ran 1ul on gel -
Conc on gel looks like ≈ 100ng/ul

digest w/ BamHI HindIII

		double digs
pMBrp4	4 ul	4
enzyme	0.5 ul	0.5 each
10X Buff E	1 ul	1
H2O	4.5 ul	4

digest from 2:00 - 5:00 37°C

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Signed

2/13/02

Ligation: To End Conversion Rx mix add:

(1) 1ul pls88 Vector (pls 88 EcoRV digest)
1ul ligase

(2) + control
1ul pT7 Blue Vector
1ul ligase

(3) - Control
1ul pT7 Blue Vector
1ul ligase

22°C 15"

~~Plate~~

Transformation:

Add 1ul ligation Rx to 1 tube of Nova Blue Comp cells

Incubate on ice 5min

heat shock 30 sec in 42°C water bath

Incubate on ice 2min

Add 250ul RT SOC Media

plate

(+) + (-) Controls 50 ul of 1/10 (5ul in 45ul H_2O)
on LB S-gal

exp 50ul of 1/10 on LB strep

2/14/02

Colonies from ligation were very small first thing this AM.

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Signed

Notebook Number: _____

Date: _____

3/14/02 Thursday

PCR pCLpm3 1.7 kb T7SalI P2 + CLdamECoRV 3/14/02			
Sample #	**1**		
pCLpm3 PCR 1.7 kb purified (1/23/02)	0.5		
dNTPs 1mM each	5		
T7Sal IP2 +CLDamECoRV	1		
Taq (7/16/01)	0.5		
Taq Buffer	5		
Water	38		
TOTAL Rx amt.	50		
Annealing temp	55		
extension time	30 sec		

used wrong template
repeat using
pCLpm3 plasmid prep
8/29/01 red box G8

15 7369-065 Mar 7 2002
GENOSYS (800) 234-5362
CLDamEcORV
5'-CTCTTTTGATATCGTCTCT TTTCA
341.8µg | Tm=80.3°C
10.00D | 34.2µg/OD
47.3nmol | MW=7225

15 7368-065 Mar 7 2002
GENOSYS (800) 234-5362
T7SalIP2
5-CCCCTGATTCTGTGGACA
514.3µg | Tm=62.4°C
15.40D | 33.4µg/OD
95.1nmol | MW=5410

reconstituted primers
2.5ml each + 95ml H2O

Made new 1mM dNTPs
40ml 10mM mix to 60ml H2O

plated pLS88 from frozen stock A3 E4 put on LB strep at 37C ML will take out of incubator + put at 4 C tomorrow

4/16/02

Hybridize colonies from pl558 PCRpm3 ligation

follow protocol pg. 50

probe

PCR prod green PCR box A9 = PCR-Nx 3/21/02
pg. 94 bkII = 5.35 ng/ul

Cleanup pcr prod using microcon columns -
follow directs in kit

removes primers & dNTPs

Re read on Gene Spec -

Type	230nm	260nm	280nm	320nm	ratio	Concentration	Dilution	Factor
dsDNA	0.709	0.366	0.224	0	1.63393	0.0183 ug/uL	1	50
dsDNA	0.736	0.655	0.497	0	1.31791	0.03275 ug/uL	1	50
dsDNA	0.492	0.945	0.804	0	1.17537	0.04725 ug/uL	1	50
dsDNA	0.551	0.997	0.861	0	1.15796	0.04985 ug/uL	1	50

These are crazy readings - gene spec is
not good for measuring DNA of PCR prod.

MLL said use 3ul in probe

Remainder of cleaned-up PCR prod is in Pink Box H8

Hybridize ON 42°C

4/17/02 Developed Hybridization

1" film almost had no image
10" very light image
1 hr OK

Plate 1

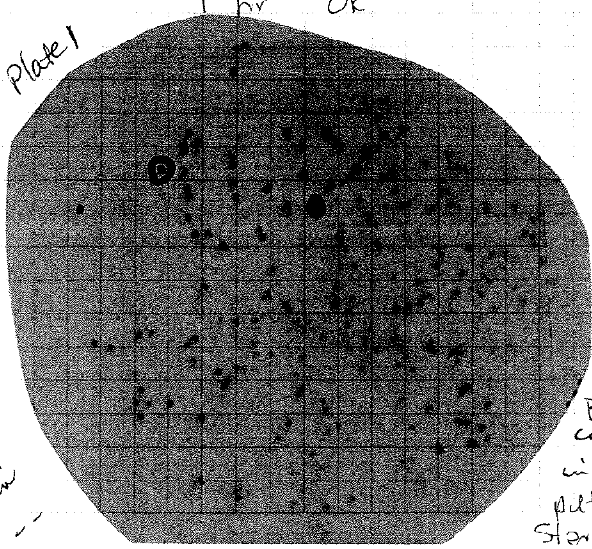
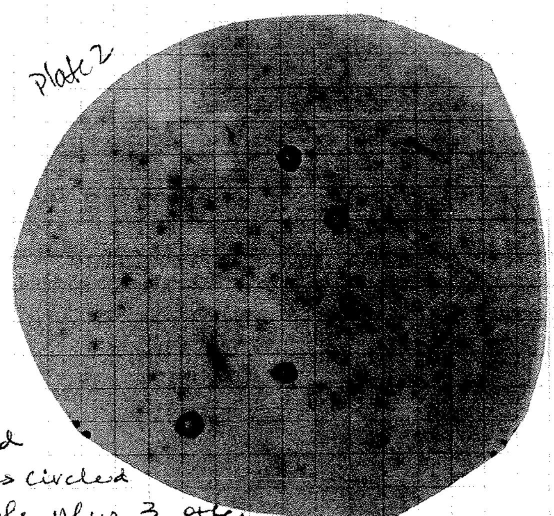


Plate 2



passed
colonies circled
in purple plus 3 other
put on LB strep

plates in ...

Start 5ml LB ... [illegible]

5/13/02

cycle seq PGEM 32 test plasmid w/ M13 primer from ABI
Run on Seq. to create Matrix file

5/14/02 Tues

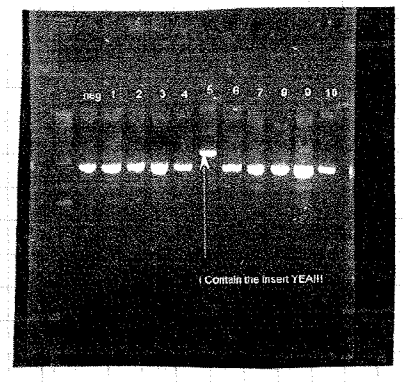
Clones from Electroporation grew well

grow 2ml LB strep cultures
pick colonies w/ sterile toothpick
use 1ml tomorrow to do plasmid preps to ✓ for insert

Grow 1 culture from Neg Control

pls88 pclpm3	100ml	≈150	53
	50ml	26	5
Neg Con (pls88 No insert)	100ml	21	~~55~~
	50ml	14	11

pick 10 ligation colonies
1 neg con colony



5/15/02 Wed

all 2ml cultures grew

used 1ml to do Qiaprep spin plasmid preps

eluted in 30ml EB

Run 3ml DNA on gel
1 ml 6X Dye
2 ml H2O
6 ml

It Finally WORKED!!

#5 d Neg Plasmid preps in yellow box A5 + A6

For Vaccine trial:
plated 93146 R6 from 6/19/01 A4 G5 -80C on Blood
grow at 26°C 48hrs

Reconstituted Intervet Edict vaccine - filled bottle 2/3 full of sterile H2O

Plate 1 loop full on Blood grow at 26 C 48hr
put 50ml into 5ml BHI to freeze back grow at 26C shaking over night

1 75Dml flask BHI for Intervet Vaccine
4 75Dml flask BHI for R6

Michelle Signed

Read and Understood By

Signed

8

Notebook Number: _____

Date: _____

For Vaccine trial cont:

Took 5mls of heat Killed Ed ict (BKR's prep 4/12/02)

Tared tube empty
add 5mls
Cf 12000rpm 10"
Aspirate Sup
Re weigh tube = 0.0499 gm
Lyophize Overnight + Re weigh

resuspended pellet in 2mls H2O
~~Freeze~~ Transfer to a pre-weighed 15 ml tube = 6.6018 gm
Freeze
Lyophilize
15 ml tube Dry wt = 6.6064

Dry wt of pellet = .0046

Digest pLs 88 pclpm3 + clone (#5) + Neg plasmid preps
w/ sal I + Cla I single digests

2ul DNA
1 ul Buffer
0.5ul Enzyme
6.5 ul H2O
10ul digest

ABI ~~maxta~~ matrix stds came in. Stds are 4 colors: Blue, Red, Green, yellow

mixed 1ul of each Standard with 12ul of deionized formamide (Borrowed from Hansons Lab) 1 tube per std = 4 tubes 1 each color

Heat to 95C for 2"
Chill on ice

Sequence using Seq Run (250ul) E run module

ingected each sample 3 times - this is done when the ingection list is filled out

5

Date: ~~- Bk 5ml culture~~ ~~- clone #5 5ml culture/freeze~~ ~~- Weigh lyophilized heat killed Ed~~ ~~- Run digests on gel (in yellow box)~~

5/16/02 Thurs

Stds are still running on seq.

Weighed lyopholized pellet of heat killed Ed. ict - Monday previous page

prepare 2-750 ml cultures of heat killed Ed. ict - Monday

Run digests on gel plr88 pclp m1 electroporate plasmid prep clone #5 into 11039 comp cells A11 D1-3 BH1 strep pls dam 2 Plate 100ul on 2 LB strep

Load entire Digest
6X dye is really conc. So only use

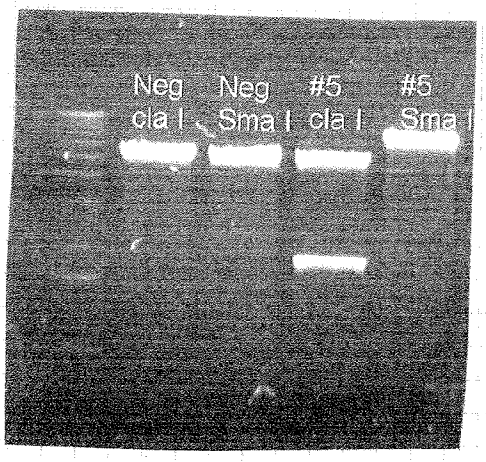
10ul DNA digested
1 ul 6X Dye
1 ul H2O

12 ul on gel

FIOT 93146 #19 pass from MW plate

0.7% ag ~~Make 2 more 750ml BH1 broth cultures~~ gel std

run 100 Volts



Lane 1 1ul 1Kb
2 Neg Cla I
3 Neg Sal I
4 #5 Cla I
5 #5 Sal I

Electroporate: pls dam 2 into 11039 p.malt. electrocompetent cells

1ul plasmid prep #5 (yellow box) A8
40ul 11039 Comp cells box A11 D2

Tc = 5.12
3.5 KV
200 Ω
25 μF

Recovered 1 hr in 1ml LB 37C

plate 100ul neat on 2 LB Strep plates

Made 2 more 750ml broth cultures to grow 93146 wt #19 to heat ~~kill~~ Kill for vaccine trial

Read and Understood By

Michelle S
Signed

37g x 750ml = 27.75 gms BH1

10

Notebook Number: _____

Date: _____

5/16/02 Thurs.

Froze

pls Dam 2 5 vials 1ml/each in LB strep + 20% glycerol

Aquavac-Esc 5 vials 1ml/each in BHI to 20% glycerol Vaccine

5/17/02 Fri.

Staff Appreciation Day.

Mothers has grown yet on plates from Electroporation -

maybe need to be on BHI strep

Make 11039 comp cells Dead

Leave at 37 C until Tomorrow

Passed 93146 WT #19 to new blood plate

Start 5ml culture Sun

Took Rec + Vaccine plates out of Incubator

Leave at RT

Start 5 ml BHI Cultures Sat.

5/19/02 - started 5ml BHI Cultures

5/20/02 MM leave Mark Sick BYA started 2 750ml cultures of ~~ES~~ WT #19

5/21/02 93146 WT Ed. ict take 1ml from each 750ml culture

Combine in 1 tube

Streak for purity - plate is pure

plate 10^{-5} + 10^{-6} for colony Counts

10^{-5}	TNTC	TNTC	
10^{-6}	286	287	Avg 286.5

Heat Kill at 60°C for 3 hours

plate for Viability - NO growth at 48 hrs

7/24/02

Blotted plates on Nitrocellulose membrane

Block in 0.01m PBS + 5% NFM 2 hrs RT

1° Ab used Straight Ed 9 1 hr RT

3X wash PBS 0.01M + Tw20

2° Ab goat α mouse Ig(H+L) AP 1/1500 4°C Overnight

Set up Serum Killing Assay see pg 17 for plate setup

Serum HI at 56°C for 30min

Read plate every 5min for 1 hr

plate 15ul of 10^{-4} & 10^{-4} on BHI Ap

Colony Count results (7/25/02)

assay worked perfectly

E. coli	10^{-6}	10^{-7}	2.31×10^9
	27, 30, 47	0, 2, 3	1.56×10^9
7/26/02 WT	10^{-6}	10^{-7}	1.82×10^9
	35, 21, 26	2, 5, 9	3.56×10^9
RC	10^{-6}	10^{-7}	2.42×10^9
	44, 30, 35	0, 3, 6	2×10^9

3

Injected mice

100ul ip. of p.multocida WT 50, 100, 1000 2 mice per dose

" Dam 50, 100, 1000

" Dam 2 50, 100, 1000

Controls PBS 3 mice

Michelle S

Signed

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Signed

24 CO_2 indicates mouse was euthanized to reduce suffering

Notebook Number: _____

Date: _____

7/25/02 Mortality from mice Exp.

9:00Am WT 1000 2 mice 1st DoA 2nd CO_2 Lethargic, Shivering, flaccid, pilo erection
WT 50 1 CO_2
Dam 1000 1 DoA
Dam 100 1 DoA
Dam 2 100 1 DoA

Notes:
2nd mouse in WT 50 Trt is very lethargic, doesn't respond to being handled
2nd mouse in Dam 1000 is lethargic + shivering (due to fever?)

11:30Am WT 100 CO_2 lethargic, Fever (shivering) does not respond to handling
Dam 1000 1 DoA
Dam 50 1 DoA

Notes:
2nd mouse in Dam 50 is sick - shivering, non-responsive, pilo erection

1:30 No morts

4:45 WT 50 1 DoA
Dam 50 1 DoA
WT 100 1 DoA

All mice from Trt Groups WT 1000, WT 100, WT 50, Dam 1000 + Dam 50 are Dead

Dead mice were opened ventrally, the chest was spread open & they were stored in Sarpet cups w/ 10% Buffered formalin.

7/26/02

9:00Am Dam 100 1 DoA

All mice from trt Group Dam 100 are Dead

7/27 + 7/28 Dan Scruggs ✓ mice

Mon 7/29/02

Start WT, R6 + E. coli Lux cultures on ~~BHI~~ + 5 mL BHI tubs both

Mice - Am all Trt groups are active + Alert
Pm " " " "

8/28/02

Colony counts- Serum Killing Assay E. coli Lux

10^{-7} 5
5
5

1.6×10^{10} CFU/ml

10^{-6} 35
51
25

3.7×10^{10} CFU/ml

8/29/02

R6 lux + WT Lux Colony Count plates were Contaminated too badly to count

9/5/02 Inject mice

B/R Prepared p. multocida 11039 WT + Dam2 for injections

1 xtra mouse uninjected

5 mice / dose 9 treatments

20 - 4 doses WT 5, 10, 50, 100

20 - 4 doses Dam 2 10, 50, 100, 500

5 Control mice - PBS

Inject mice w/ 100ul each i.p.

use 1cc syring with the 27 1/2 G needle

mice were not set up properly - we (Dan Scruggs + I) had to redistribute mice to 5/cage before injection — mice were Stressed!

Injected mice 11:30am ✓1:30 pm All OK ✓4:15pm all OK

Read and Understood By

Signed

Signed

Notebook Number: _____

Date: _____

9/6/02

✓mice 8:00 Am

WT 5 - 3 mortalities
1 lethargic, shivering, unresponsive
1 Almost dead completely unresponsive, unable to walk euthanize w/ CO_2
→ dead before I could euthanize

WT 10 - 4 morts
1 sluggish but responsive

WT 50 3 mort
2 sluggish but responsive

WT 100 3 mort
2 sluggish but responive have pilo erection

Dan2 500 All OK
100 all OK
50 all OK
10 all OK

PBS (-) Control All OK

✓mice 10:45 Am

WT-100 both mice are shivering, lethargic, very rapid breathing

✓mice 2:45 pm

WT 100 breathing very rapidly unresponsive
WT 50 " "
WT 10 very lethargic
WT 5 Sick but alert

Dan2 - all alert but not as alert as controls

✓mice 6:20 pm

WT No change
Dan2 No change
PBS No change

Notebook Number: _____

Date: _____

10-4-02

Actinobacillus - plasmid electroporation

1- Removed 40 µl of actinobacillus competent cells from microfuge tube and added to a clean tube.
2- Added 1µl of plasmid DNA to the same tube.
3- Placed suspension in electroporation cuvette.
4- Set electroporator settings.
5. Electroporated and got a time constant = 5.26.
6- Placed cells suspension in 1 mL BHI_{NAD}^{+}.
7- Incubated for 1 hour at 37°C.
8- Diluted culture to 10^{-2}.
9- Plated 100 µl of 10^{0}, 10^{-1}, 10^{-2} on BHI_{NAD}^{+} strept plates.
10- Incubated plates at 37°C overnight.

Results

10-5-02

	Plate Count
10^{0}	33
10^{-1}	8
10^{-2}	0

10-29-02

Mutation Rates

1- Diluted XL1 Blue, XL1 dam-, Actin, and Actin dam- to 10^{-6}.
2- Plated in triplicate with 10µL of 10^{-5} and 10^{-6}.
3- Plated 100µL of overnight culture in triplicate on BHI,f. 100µg/mL or BHINAB,f 100µg/mL.
4- Incubated at 37°C overnight.

Results

Plate counts	10^{-5}	10^{-6}
XL1 B	TNTC	TNTC
XL1 dam-		
Actino		
Actino dam-		

BHI,f

XL1	Bacterial lawn
XL1 dam-	Bacterial lawn
Actin-	No growth
Actino dam-	No growth

Note: Will have to do antibiotic titer with Actinobacillus and rifampicin

10.29.02

Fish Trail (24 hour)

1- Pooled 3 fish from each tank.
2- Removed spleen and kidney.
3- Weighed, macerated, diluted to 10^{-2}.
4- Plated 15µL of 10^{0}, 10^{-1}, 10^{-2} on EIM plates.
5- Incubated at 30°C for 48 hours.

1. Shaken cultures of HB101 30.96 donor electroporation for plasmid preps.
2. Shaken cultures of 93146 WT, Rc, and EC 11229 for MIC micro.

Killing with conjugation mutants

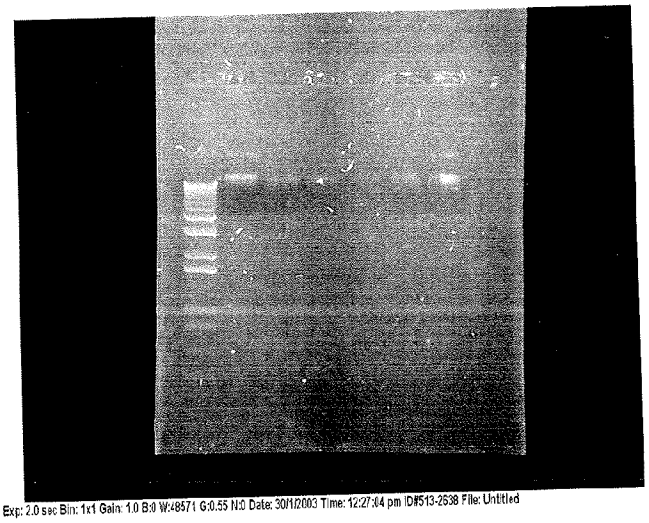
1. Pelleted bacteria in microtiter plate.
2. Resuspended in 100 µl HBSS 0.9X with Ampicillin.
3. Removed 5µl from resuspended plate and added to 295µl HBSS amp.
4. Removed 5µl from the dilution plate and added to 35µl HBSS amp in white bottom assay plate.
5. Added an additional 40µl HBSS to dilution plate and 40µl normal serum to assay plate.
6. Incubated at RT for 1 hour.
7. Took readings on Dmax at Time 0, 30, and 60.
8. Analyzed data in Excel.
9. Highlighted mutants were frozen back (-80°C).

Results: See next page. (86)

1.30.03 HB101 30.96 donor plasmid prep.

1. Removed plasmid DNA with Qiagen kit.
2. Loaded 3µl on 1% agarose gel.
3. Ran at 100V for 1 hours.

Lane 1 - 1 Kb ladder
Lane 2 - prep 1
Lane 3 - prep 2
Lane 4 - prep 3
Lane 5 - prep 4
Lane 6 - prep 5
Lane 7 - prep 6



Signed

Signed